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We define a novel mechanism by which integrins regulate growth factor expression and the survival of carcinoma cells. Specifically, we demonstrate that the α6β4 integrin enhances VEGF translation in breast carcinoma cells. The mechanism involves the ability of this integrin to stimulate the phosphorylation and inactivation of 4E-Binding Protein (4E-BP1), a translational repressor that inhibits the function of translation initiation factor 4E (eIF-4E). The regulation of 4E-BP1 phosphorylation by α6β4 derives from the ability of this integrin to activate the PI-3K/Akt pathway and, consequently, the rapamycin-sensitive kinase mTOR that can phosphorylate 4E-BP1. Importantly, we show that this α6β4-dependent regulation of VEGF translation plays an important role in the survival of metastatic breast carcinoma cells by sustaining a VEGF autocrine signaling pathway that involves activation of PI3-K and Akt. These findings reveal that integrin-mediated activation of PI-3K/Akt is amplified by integrin-stimulated VEGF expression and they provide a mechanism that substantiates the reported role of α6β4 in carcinoma progression.

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Introduction

An understanding of the mechanisms that sustain the survival of tumor cells in adverse physiological conditions is one of the most important problems in cancer biology and metastasis. The overall goal of our proposal was to define the role of integrin ($\alpha6\beta4$) and its activation of Akt in the metastasis of breast carcinoma cells focusing their survival mechanism against hostile condition. Since VEGF appears to be an essential factor for the survival and progression of many solid tumors (Bachelder et al., 2001; Brown et al., 1999; Dvorak et al., 1999; Shweiki et al., 1992) and the $\alpha6\beta4$ integrin can promote the survival of breast carcinoma cells in stress conditions (Bachelder et al., 1999), we tested the hypothesis the potential regulation of VEGF expression via $\alpha6\beta4$ integrin. We detected a significant influence of $\alpha6\beta4$ on VEGF translation and protein expression in these cells, an observation that reveals the ability of this integrin to regulate translation. The mechanism by which $\alpha6\beta4$ regulates VEGF expression involves its ability to stimulate the phosphorylation of 4E-Binding Protein (4E-BP1) in a PI-3K and Akt dependent manner. Phosphorylation of 4E-BP1 by mTOR, a downstream effector of Akt, disrupts its binding to translation initiation factor eIF-4E, which is present in rate-limiting amounts in most cells (De Benedetti and Harris, 1999; McKendrick et al., 1999). The regulation of 4E-BP1 phosphorylation by $\alpha6\beta4$ derives from the ability of this integrin to activate the PI-3K/Akt pathway and, consequently, mTOR. Our findings reveal a novel mechanism of tumor cell survival and they highlight the ability of a specific integrin to regulate protein translation by influencing eIF-4E activity.

Body

Following are the accomplishments associated with our Statement of work. These works were published in Cancer Research (Bachelder et al, 2001) and in press for publication in Journal of Cell Biology (Chung et al, July 8^{th,} 2002). We attached the reprint of Cancer Research manuscript and preprint of Journal of Cell Biology manuscript to this report and refer the figures below in the attached manuscript.

1) **Examine the hypothesis that the α6 integrins promote the survival of metastatic breast carcinoma cells in adverse conditions by regulating Akt signaling pathway.**

We have used the MDA-MB-435 breast carcinoma cell line which does not express α6β4 integrin and its stable subclones that express the intact α6β4 integrin to address the role of this integrin in survival under low serum condition. A significant level of apoptosis was observed following 24 hours of serum deprivation in the parental MDA-MB-435 cells and mock transfectants, as well as in transfectants that express α6β4 containing a cytoplasmic domain deletion of the β4 subunit that lacks the ability to signal (Shaw et al., 1997)(Fig. 1A, chung et al, 2002). Stable subclones that express the intact α6β4 integrin, however, were protected from apoptosis under these conditions. Based on these results and our previous finding that the survival of metastatic breast carcinoma cells is dependent on VEGF, we used a VEGF antisense oligonucleotide to reduce VEGF expression in the MDA-MB-435/β4 transfectants and assessed the impact of reducing VEGF expression on their survival (Fig. 1B and C, Chung et al, 2002). The VEGF

antisense oligonucleotide reduced VEGF protein expression significantly in the β4 transfectants (Fig. 1C, Chung et al, 2002). Therefore, the reduction in VEGF expression abrogated the survival enhancing effect of α6β4 under conditions of serum-deprivation.

We further demonstrated that α6β4 integrin increase VEGF protein level without affecting VEGF mRNA level (Fig. 2A and B, Chung et al 2002) suggesting that regulation occur at the level of VEGF translation. To substantiate the regulation of VEGF expression by α6β4, integrin-specific antibodies were used to cluster either α6β4 or α5β1 and the effects of integrin-mediated clustering on VEGF expression were assessed by immunoblotting. A substantial induction of VEGF expression was observed upon α6β4 integrin clustering in the β4 transfectants but not in the mock transfectants, and no induction was seen in response to α5β1 clustering (Fig. 2C, Chung et al 2002). Importantly, the induction of VEGF expression that occurs in response to α6β4 clustering was inhibited by cycloheximide (Fig. 2C). This result together with the real-time PCR data (Fig. 2A) and polysome analysis of the VEGF message (Fig. 3, Chung et al) indicates that α6β4 is influencing VEGF translation.

The α6β4 integrin is a potent activator of the PI-3K/Akt signaling pathway in MDA-MB-435 and other carcinoma cells (Bachelder et al., 1999a; Gambaletta et al., 2000; Hintermann et al., 2001; Nguyen et al., 2000; Shaw et al., 1997), and this pathway has been linked to the regulation of protein translation. Specifically, the serine/threonine kinase mTOR is activated by Akt-mediated phosphorylation events (Sekulic et al., 2000). Phosphorylation of 4E-BP1 by mTOR disrupts its binding to eIF-4E, enabling eIF-4E to initiate translation of VEGF and other molecules (De Benedetti and Harris, 1999). We hypothesized, based on this information, that α6β4 regulates 4E-BP1 phosphorylation

and, as a consequence, VEGF expression. Initially, we assessed the steady state phosphorylation levels of 4E-BP1 and S6 kinase ($p70^{S6K}$), which are both downstream targets of mTOR, in cells that had been serum-deprived for 24 hours. Indeed, a marked increase in the level of phosphorylation of 4E-BP1 (on Ser65) and $p70^{S6K}$ (on Thr389) was evident in the MDA-MB-435/β4 transfectants relative to either the mock transfectants or the parental cells (Fig. 4A, chung et al, 2002). Phosphorylation of Ser65 of 4E-BP1 has been shown to be critical for dissociation of 4E-BP from eIF-4E (Gingras et al., 2001a). To confirm the specificity of the α6β4 integrin in mTOR signaling, the effects of integrin-mediated clustering on 4E-BP1 phosphorylation were assessed. A substantial induction of Akt, 4E-BP1 and $p70^{S6K}$ phosphorylation was observed upon α6β4 integrin clustering in the β4 transfectants but not in the mock transfectants (Fig. 4C, Chung et al 2002). In contrast, clustering of the α5β1 integrin did not stimulate phosphorylation of these molecules in either the mock or β4 transfectants. Collectively, these data demonstrate the preferential ability of the α6β4 integrin to regulate the mTOR signaling pathway and, more importantly, the phosphorylation of 4E-BP1.

To establish a role for α6β4 in regulating VEGF expression and survival rigorously, we used a small interfering RNA (RNAi) approach to inhibit β4 expression in MDA-231 cells which endogenously express α6β4. RNAis specific for the β4 subunit and the corresponding inverted sequence were designed and expressed in these cells by transfection. The cells were maintained in low serum (0.5%) for 24 hours post-transfection and then analyzed. As shown in Fig. 8A (Chung et al, 2002), cells transfected with the RNAi specific for β4 exhibited a significant reduction in β4 expression in comparison to either untransfected cells or cells transfected with the inverted sequence.

Importantly, the reduction in β4 expression by RNAi coincided with a marked reduction in 4E-BP1 phosphorylation and in the steady-state level of VEGF (Fig. 8B, Chung et al, 2002), as well as an approximate three-fold increase in annexin V staining (Fig. 8C, Chung et al, 2002). These results link α6β4 expression directly to 4E-BP1 phosphorylation VEGF expression and survival in a carcinoma cell line that expresses endogenous α6β4.

Subsequently, we performed antibody clustering experiments to substantiate the regulation of VEGF expression by α6β4 (Fig. 8D, Chung et al, 2002). Clustering of the α6β4 integrin with either an α6 integrin specific antibody (mAb 2B7) or a β4 integrin specific antibody (mAb A9) stimulated the phosphorylation of 4E-BP1 and Akt, and increased VEGF expression. In contrast, no induction of VEGF expression or stimulation of either 4E-BP1 or Akt phosphorylation was observed upon clustering with an α5 integrin specific antibody (mAb Sam1) or IgG.

2) **Examine the hypothesis that the Akt-dependent survival of breast carcinoma cells in adverse conditions (anoikis and hypoxia) is linked to metastatic potential.**

The hypothesis that activation of Akt is a major determinant for the stimulation of 4E-BP1 phosphorylation and VEGF expression was assessed by expressing a constitutively active Akt construct in MDA-MB-435 cells that are deficient in α6β4 signaling. For this purpose, we used MDA-MB-435/mock transfectants that lack α6β4 expression and the MDA-MB-435/β4 Y1494F transfectants, described above, which are deficient in α6β4-mediated activation of PI-3K. These cells were infected with adenoviruses that encoded

either a myristoylated Akt (Myr-Akt) construct or β-galactosidase as a control. Expression of Myr-Akt stimulated 4E-BP1 phosphorylation and VEGF expression substantially in both populations of transfectants in comparison to cells that expressed β-galactosidase (Fig. 7, Chung et al, 2002). This result indicates the critical importance of Akt activation by α6β4 for stimulating VEGF expression. Expression of Myr-Akt also enhance MDA-MB-435 cell survival as well against serum starvation (data not shown) suggesting a critical role of Akt in tumor cell survival by regulating VEGF protein expression.

3) **Examine the hypothesis that α6β4 integrin and Akt signaling are critical for breast carcinoma metastasis *in vivo*.**

All the data presented above suggested the critical role of α6β4 integrin and Akt signaling pathway in breast carcinoma progression. Therefore, we will establish the mouse model injected with tumor cells which lack α6β4 integrin or transfected with dominant negative Akt. More specifically, Stable transfectants of metastatic breast carcinoma cells (MDA-MB-435 and 231) will be generated by expressing short hairpin (sh) RNA specific for the β4 integrin subunit and assess the impact of reduced α6β4 expression on their behavior such as invasion and survival *in vitro* and eventually mestastic progression *in vivo* . We also plan to generate stable transfectants of MDA-MB-231/435 cells with dominant negative Akt in a tetracycline regulated vector. All these transfectants will be injected to mammary fat pad of *nu/nu* mice and weight of primary tumor and frequency of lung metastases will be measured.

Key Research Accomplishment

- We discovered a novel mechanism by which integrins regulate growth factor expression. Specifically, our findings demonstrate the ability of a specific integrin ($\alpha6\beta4$), which has been implicated in carcinoma progression (Mercurio and Rabinovitz, 2001), to stimulate the translation of VEGF and sustain a VEGF autocrine loop that is essential for survival.

- We defined the signaling pathway regulated by $\alpha6\beta4$ that involves the preferential ability of this integrin to stimulate the phosphorylation of 4E-BP1 by activating the PI-3K/Akt pathway.

Reportable Outcomes

Manuscripts:

1. Mercurio AM, Bachelder RE, **Chung J,** Oconnor K, Rabinovitz I, and Shaw LM. Integrin Laminin Receptors and Breast Carcinoma Progression. **Journal of Mammary Gland Biology and Neoplasia 2001**, 6: 299-309

2. Bachelder RE, Crago A, **Chung J**, Wendt M, Shaw L, Robinson G, and Mercurio AM. VEGF is an autocrine survival factor for neuropilin-expressing breast carcinoma cells. **Cancer Research 2001**, 61: 5736-5740

3. **Chung J,** Bachelder RE, Shaw LM, and Mercurio AM. Integrin regulation of eIF-4E

activity and VEGF translation. (**Journal of Cell Biology 2002, in press**)

Abstracts:

1. **Chung J,** Bachelder RE, Shaw LM, and Mercurio AM. Integrin regulation of eIF-4E activity and VEGF translation. BIDMC, Research Day 2001, Boston, MA 2001

2. **Chung J,** Bachelder RE, Shaw LM, and Mercurio AM. Integrin regulation of eIF-4E activity and VEGF translation. Keystone Symposia, Alberta, Canada 2002

Conclusion

Our data extend earlier reports on the involvement of eIF-4E, VEGF, and $\alpha6\beta4$ in carcinoma progression by linking these molecules in a common signaling pathway which involves PI-3K/ Akt and promotes tumor survival. Furthermore, they reveal a role for integrins in regulating growth factor expression by stimulating protein translation. Considering the role of $\alpha6\beta4$ integrin in the progression of many solid tumors, our finding that $\alpha6\beta4$ can induce the translational function of eIF-4E by regulating the phosphorylation of 4E-BP1 provides one mechanism to account for the role of this integrin in cancer and leads to potential anti-tumor therapeutics.

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Integrin (α6β4) regulation of eIF-4E activity and VEGF translation: a survival mechanism for carcinoma cells

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We define a novel mechanism by which integrins regulate growth factor expression and the survival of carcinoma cells. Specifically, we demonstrate that the α6β4 integrin enhances vascular endothelial growth factor (VEGF) translation in breast carcinoma cells. The mechanism involves the ability of this integrin to stimulate the phosphorylation and inactivation of 4E-binding protein (4E-BP1), a translational repressor that inhibits the function of eukaryotic translation initiation factor 4E (eIF-4E). The regulation of 4E-BP1 phosphorylation by α6β4 derives from the ability of this integrin to activate the PI-3K–Akt pathway and, consequently, the rapamycin-sensitive kinase mTOR that can phosphorylate 4E-BP1. Importantly, we show that this α6β4-dependent regulation of VEGF translation plays an important role in the survival of metastatic breast carcinoma cells by sustaining a VEGF autocrine signaling pathway that involves activation of PI-3K and Akt. These findings reveal that integrin-mediated activation of PI-3K–Akt is amplified by integrin-stimulated VEGF expression and they provide a mechanism that substantiates the reported role of α6β4 in carcinoma progression.

Introduction

An understanding of the mechanisms that sustain the survival of tumor cells in adverse physiological conditions is one of the most important problems in cancer biology. As argued recently, cancer progression is an evolutionary process that selects for cells that exhibit the capacity for survival in environmental conditions not present in normal tissue (Fearon, 1999; Hanahan and Weinberg, 2000). One survival strategy used by tumor cells is the secretion of proteins that elicit an angiogenic response, such as vascular permeability factor or vascular endothelial growth factor (VEGF).* VEGF appears to be an essential factor for the progression of many solid tumors (Shweiki et al., 1992; Brown et al., 1999; Dvorak et al., 1999). It is widely assumed that the function of VEGF produced by tumor and tumor stromal cells is to stimulate angiogenesis by acting in a paracrine fashion on vicinal endothelium (Hanahan and Folkman, 1996; Brown et al., 1999). Another mechanism for tumor cell survival is the establishment of autocrine signaling loops that act on tumor cells directly (Scotlandi et al., 1996; Tokunou et al., 2001; Wong et al., 2001). Although the significance of this mechanism has been overshadowed by angiogenesis, recent studies have substantiated the importance and necessity of such signaling loops for tumor survival (Scotlandi et al., 1996; Bachelder et al., 2001; Tokunou et al., 2001; Wong et al., 2001). Indeed, this mechanism probably contributes to the ability of cells to survive in hypoxic, poorly vascularized regions of tumors. In this direction, we described recently the existence of a VEGF autocrine signaling pathway in metastatic breast carcinoma cells that is essential for their survival (Bachelder et al., 2001).

An important issue that arises from the contribution of VEGF autocrine signaling to tumor survival is an understanding of the mechanisms that regulate VEGF expression. Such mechanisms are important not only for VEGF signaling in tumor cells, but also for angiogenesis as well. Clearly, hypoxia is a strong inducer of VEGF transcription and mRNA stability (von Marschall et al., 2001), but other factors are likely to be involved. Of note, our finding that the α6β4 integrin can promote the survival of breast carcinoma cells in stress conditions is intriguing (Bachelder et al., 1999b) and raised the novel possibility that a specific integrin, which has been implicated in cancer progression, could regulate VEGF expression. This possibility is substantiated by the finding reported here that the ability of the α6β4 integrin to promote survival is VEGF dependent.

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*Abbreviations used in this paper: 4E-BP1, 4E-binding protein; eIF-4E, eukaryotic initiation factor-4E; mTOR, mammalian target of rapamycin; Myr-Akt, myristoylated Akt; PI, propidium iodide; PI-3K, phosphatidylinositol 3-kinase; RNAi, small interfering RNA; VEGF, vascular endothelial growth factor.

Key words: integrin; VEGF; translation; carcinoma; eIF-4E

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The results described above prompted us to investigate the relationship between the α6β4 integrin and VEGF expression. We observed that the expression and signaling properties of this integrin have no impact on steady-state VEGF mRNA levels. Surprisingly, however, we detected a significant influence of α6β4 on VEGF translation and protein expression in these cells, an observation that reveals the ability of this integrin to regulate translation. The mechanism by which α6β4 regulates VEGF expression involves its ability to stimulate the phosphorylation of 4E-binding protein (4E-BP1). 4E-BP1 is phosphorylated by mammalian target of rapamycin (mTOR), a protein kinase whose catalytic domain is structurally related to that of phosphatidylinositol 3-kinase (PI-3K) (Dennis et al., 1999; Schmelzle and Hall, 2000). Phosphorylation of 4E-BP1 by mTOR disrupts its binding to eukaryotic translation initiation factor eIF-4E, which is present in rate-limiting amounts in most cells (De Benedetti and Harris, 1999; McKendrick et al., 1999). eIF-4E plays a critical role in the recruitment of the translational machinery to the 5′ end of mRNA, which is demarcated by an m7GpppN cap (where m is a methyl group and N is any nucleotide) (Raught and Gingras, 1999). The m7 cap is essential for the translation of most mRNAs including VEGF (De Benedetti and Harris, 1999; Raught and Gingras, 1999). Dissociation of 4E-BP1 from eIF-4E enables eIF-4E to initiate translation (Gingras et al., 1999, 2001b). The regulation of 4E-BP1 phosphorylation by α6β4 derives from the ability of this integrin to activate the PI-3K–Akt pathway and, consequently, mTOR. Our findings reveal a novel mechanism of tumor cell survival and they highlight the ability of a specific integrin to regulate protein translation by influencing eIF-4E activity.

Results

The ability of the α6β4 integrin to promote the survival of carcinoma cells is VEGF dependent

To examine the hypothesis that the ability of the α6β4 integrin to promote survival is VEGF dependent, we used MDA-MB-435 cells, which lack expression of this integrin. Stable expression of α6β4 in these cells enhances their ability to survive in stressful conditions (Bachelder et al., 1999b). Importantly, however, expression of α6β4 does not alter the expression of other integrin subunits in these cells and does not influence their adhesion to matrix (Shaw et al., 1997). As shown in Fig.1 A, a significant level of apoptosis was observed after 24 h of serum deprivation in the parental MDA-MB-435 cells and mock transfectants, as well as in transfectants that express α6β4 containing a cytoplasmic domain deletion of the β4 subunit that lacks the ability to signal (Shaw et al., 1997). Stable subclones that express the intact α6β4 integrin, however, were protected from apoptosis under these conditions. Based on these results and our previous finding that the survival of metastatic breast carcinoma cells is dependent on VEGF, we used a VEGF antisense oligonucleotide to reduce VEGF expression in the MDA-MB-435/β4 transfectants and assessed the impact of reducing VEGF expression on their survival (Fig. 1, B and C). The VEGF antisense oligonucleotide reduced VEGF protein expression significantly in the β4 transfectants (Fig. 1 C). As shown in Fig. 1 B, this reduction in VEGF expression abrogated the survival-enhancing effect of α6β4 under conditions of serum deprivation.

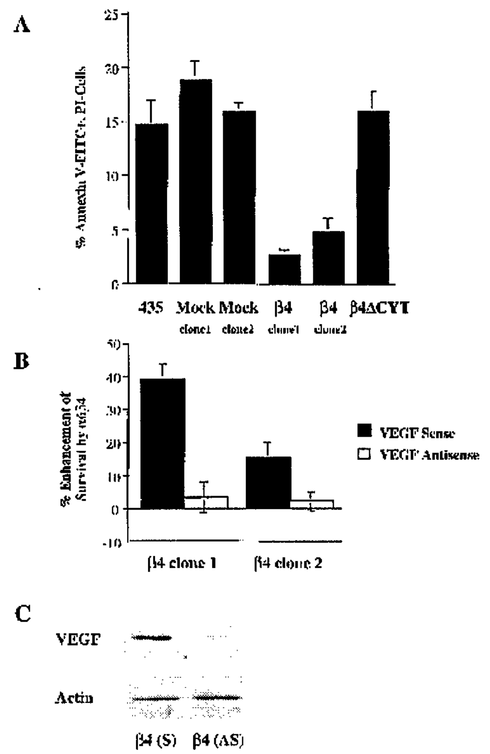

Figure 1. **The α6β4-mediated survival of breast carcinoma cells is VEGF dependent.** (A) Parental, mock (clone 1, 6D2; clone 2, 6D7), β4-ΔCYT–expressing (cytoplasmic tail deletion mutant), and β4 integrin–expressing (clone 1, 3A7; clone 2, 5B3) MDA-MB-435 subclones were maintained in low serum (0.5% FBS) medium for 24 h. To assess the level of apoptosis, these cells were stained with annexin V–FITC and propidium iodide (PI), and analyzed on a Becton Dickinson flow cytometer using CellQuest software. The percentage of annexin-positive, PI-negative cells (± SD) is indicated. Results were obtained from three independent experiments. Apoptosis was minimal in the presence of 10% FBS (unpublished data). (B) Mock-transfected clone 6D7 and β4 integrin– (clone 1, 3A7; clone 2, 5B3) expressing MDA-MB-435 subclones were transiently transfected with VEGF sense or antisense oligonucleotides and maintained in low serum (0.5% FBS) medium. After 24 h, the level of apoptosis in these cells was assessed as described above. The data are presented as the mean difference (± SD) in annexin positivity between mock-transfected and α6β4-expressing MDA-MB-435 cells. Similar results were observed in two separate experiments. (C) The relative amount of VEGF protein in extracts obtained from the MDA-MB-435/β4 cells transfected with either the VEGF sense (S) or antisense (AS) oligonucleotide was determined by immunoblotting using a polyclonal anti-VEGF immune serum.

The α6β4 integrin increases VEGF protein but not mRNA expression

Given that the survival effect of α6β4 expression is VEGF dependent, the novel possibility arose that VEGF expression could be regulated by this integrin. VEGF expression can be regulated at the level of both transcription and mRNA stability (Nabors et al., 2001; von Marschall et al., 2001), mechanisms that would alter the steady-state levels of VEGF

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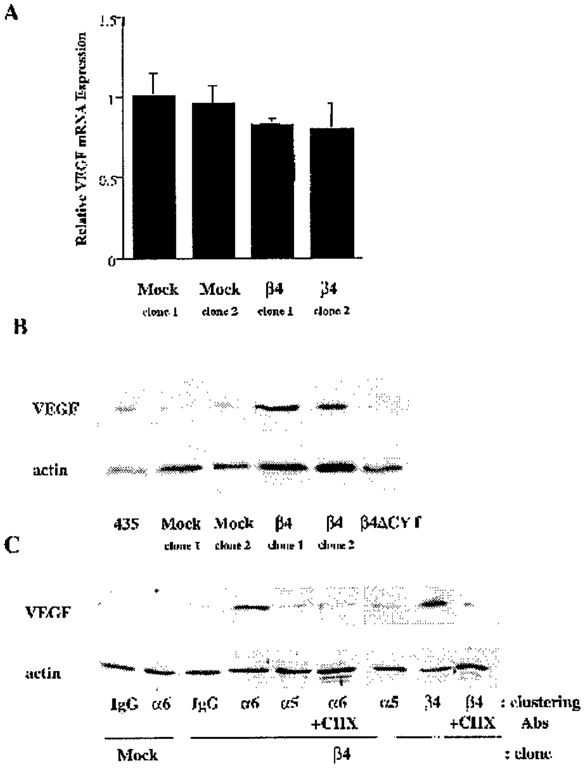

Figure 2. **Expression of the α6β4 integrin increases VEGF protein but not steady-state mRNA.** (A) The amount of VEGF mRNA in extracts obtained from mock- (clone 1, 6D2; clone 2, 6D7) and β4 integrin– (clone 1, 3A7; clone 2, 5B3) transfected MDA-MB-435 subclones was quantified by real-time PCR. The data are presented as the mean ratio of VEGF to β-actin mRNA (± SD) obtained from triplicate samples. (B) Parental (435), mock (clone 1, 6D2; clone 2, 6D7), β4-ΔCYT–expressing (clone 1E10), and β4 integrin–expressing (clone 1: 3A7, clone 2: 5B3) MDA-MB-435 subclones were cultured in low serum (0.5% FBS) medium for 24 h. Extracts of these cells containing equivalent amounts of protein were analyzed for their relative expression of VEGF and actin by immunoblotting. Similar results were observed in four independent experiments. (C) Mock (clone 6D7) and β4 integrin–expressing (clone 3A7) MDA-MB-435 subclones were maintained in low serum (0.5% FBS) medium for 24 h. These cells were detached with trypsin and incubated with integrin-specific antibodies (α6 integrin, 2B7; β4 integrin, A9; α5 integrin, Sam1) or IgG for 30 min in suspension and allowed to adhere on anti-IgG–coated plates for 60 min before lysis. In addition, cells were preincubated in cycloheximide (CHX) at a concentration of 10 μg/ml for 30 min and then incubated with either the α6 or β4 integrin antibodies in the presence of cycloheximide. Extracts of these cells containing equivalent amounts of protein were analyzed for their relative expression of VEGF and actin by immunoblotting. Similar results were observed in two independent experiments.

mRNA. In addition, regulation can also occur at the level of VEGF translation (Kevil et al., 1996; Akiri et al., 1998; Stein et al., 1998). As shown in Fig. 2 A, quantitative analysis of VEGF mRNA levels in two clones of MDA-MB-435/mock and β4 transfectants using real-time PCR revealed no significant difference in the steady-state mRNA levels in these two populations. However, we detected a substantial increase in VEGF protein expression in the MDA-MB-435/β4 transfectants relative to either the parental cells, mock transfectants, or cells that express a cytoplasmic domain deletion of the β4 subunit (β4-ΔCYT) (Fig. 2 B). These results indicate that the α6β4 integrin regulates VEGF protein expression. It is also worth noting that the level of apoptosis observed in these populations in response to serum deprivation correlates inversely with their expression of VEGF (Fig. 1 A and Fig. 2 B).

To substantiate the regulation of VEGF expression by α6β4, integrin-specific antibodies were used to cluster either α6β4 or α5β1 and the effects of integrin-mediated clustering on VEGF expression were assessed by immunoblotting. Of note, the MDA-MB-435/β4 transfectants express equivalent levels of α6β4 and α5β1 (unpublished data). An α6-specific antibody (mAb 2B7) was used to cluster the α6β1 integrin in the mock transfectants and the α6β4 integrin in the β4 transfectants, a β4-specific antibody (mAb A9) was used to cluster the α6β4 integrin in the β4 transfectants, and an α5-specific antibody (mAb Sam1) was used to cluster α5β1 in both the mock and β4 transfectants. A substantial induction of VEGF expression was observed upon α6β4 integrin clustering in the β4 transfectants but not in the mock transfectants, and no induction was seen in response to α5β1 clustering (Fig. 2 C). Importantly, the induction of VEGF expression that occurs in response to α6β4 clustering was inhibited by cycloheximide (Fig. 2 C). This result, together with the real-time PCR data (Fig. 2 A), indicates that α6β4 is influencing VEGF translation.

To obtain more definitive evidence that α6β4 is regulating VEGF translation, we performed polysome analysis of the VEGF message. mRNA isolated from the MDA-MB-435/mock and β4 transfectants was fractionated on a sucrose gradient (Fig. 3 A) and the relative amount of VEGF mRNA in each fraction was determined by real-time PCR (Fig. 3 B). As shown in Fig. 3 B, a striking difference in the distribution of VEGF mRNA was evident in the two populations of cells. In the MDA-MB-435/β4 transfectants, VEGF mRNA fractionated in the heavy polysomal region, whereas in the mock transfectants, the majority of VEGF mRNA was associated with light polysomal to ribosomal subunit fractions. This result indicates that the translation of VEGF in the MDA-MB-435/β4 transfectants is cap dependent.

Identification of an α6β4 integrin–mediated signaling pathway that regulates VEGF expression

Our finding that α6β4 regulates the cap-dependent translation of VEGF prompted us to assess the ability of this integrin to stimulate the activity of the eIF-4E translation initiation factor. The α6β4 integrin is a potent activator of the PI-3K–Akt signaling pathway in MDA-MB-435 and other carcinoma cells (Shaw et al., 1997; Bachelder et al., 1999a; Gambaletta et al., 2000; Nguyen et al., 2000; Hintermann et al., 2001), and this pathway has been linked to the regulation of protein translation. Specifically, the serine/threonine kinase mTOR is activated by Akt-mediated phosphorylation events (Sekulic et al., 2000). Phosphorylation of 4E-BP1 by mTOR disrupts its binding to eIF-4E, enabling eIF-4E to initiate translation of VEGF and other molecules (De Benedetti and Harris, 1999). We hypothesized, based on this information, that α6β4 regulates 4E-BP1 phosphorylation and, as a consequence, VEGF expression. Initially, we as-

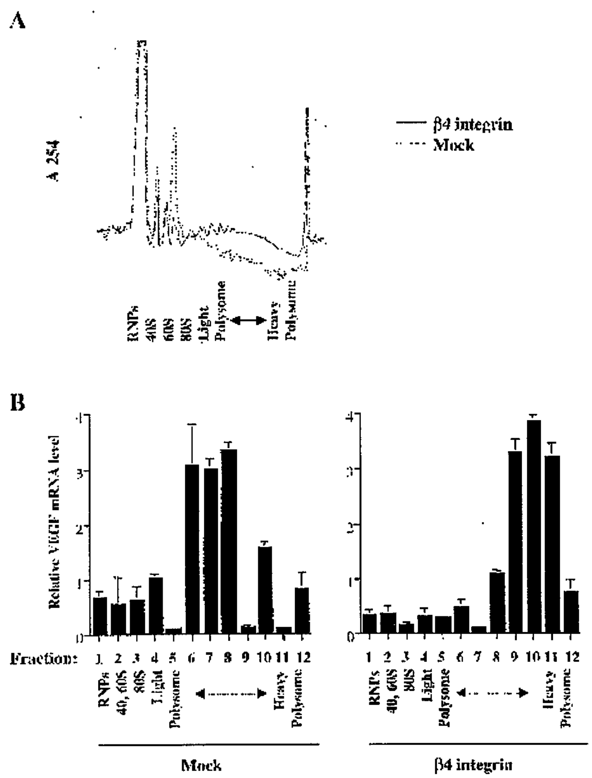

Figure 3. **Polysome analysis of VEGF mRNA.** (A) The distribution of RNA from MDA-MB-435/β4 and mock transfectants that had been fractionated on sucrose gradients as described in the Materials and methods was determined by measuring the A_{254} of each fraction. (B) The relative VEGF mRNA content of each sucrose gradient fraction was measured by real-time PCR as described in the Materials and methods. Fraction 1 contains unbound RNA present in the ribonucleoprotein fraction, fraction 2 contains 40S and 60S monosomes, fraction 3 contains 80S monosomes, fractions 4–7 contain light polysomes, and fractions 8–12 contain heavy polysomes. The data are presented as the mean ratio of VEGF to β-actin mRNA (± SD) obtained from triplicate samples. Similar results were obtained from three independent experiments.

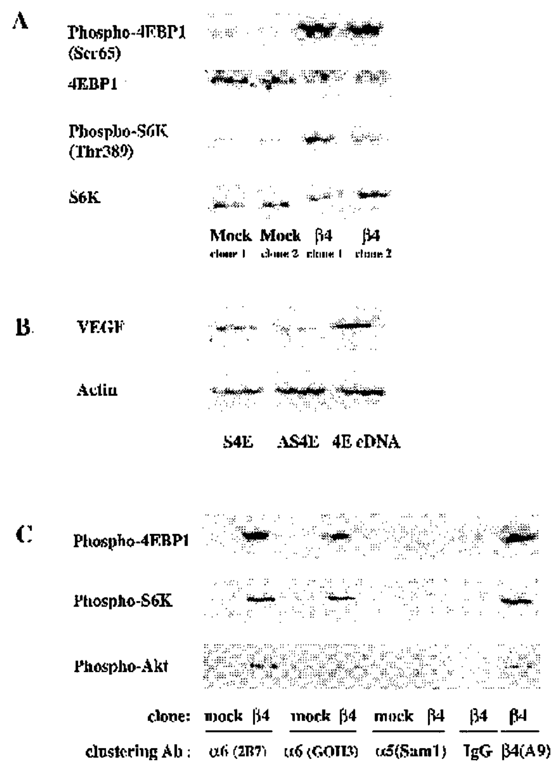

Figure 4. **The α6β4 integrin stimulates the phosphorylation of Akt, 4E-BP1, and p70^{S6K}.** (A) MDA-MB-435 parental cells, mock transfectants, and β4 transfectants were maintained in medium containing low serum (0.5% FBS) for 24 h. The phosphorylation status of 4E-BP1 on Ser 65 and S6K on Thr 389 was assessed in extracts from these cells using phosphospecific antibodies as described in the Materials and methods. In addition, the total amount of 4E-BP1 and p70^{S6K} in these extracts was assessed by immunoblotting. (B) The MDA-MB-435/β4 cells were transiently transfected with either an eIF-4E sense (S) or antisense (AS) oligonucleotide, or a full-length eIF-4E cDNA (4E). Extracts of these cells containing equivalent amounts of protein were analyzed for their relative expression of VEGF and actin by immunoblotting. (C) MDA-MB-435 mock (clone 6D7) and β4 (clone 3A7) transfectants were maintained in low serum (0.5% FBS) medium for 24 h. These cells were detached with trypsin and incubated with integrin-specific antibodies (α6 integrin, 2B7; α6 integrin, GOH3; α5 integrin, Sam1; β4 integrin, A9) or IgG for 30 min as described in the legend to Fig. 2. The phosphorylation status of 4E-BP1 (Ser 65), S6K (Thr 389), and Akt (Ser 473) was assessed in extracts from these cells using phosphospecific antibodies. Similar results were observed in four independent experiments.

sessed the steady-state phosphorylation levels of 4E-BP1 and S6 kinase (p70^{S6K}), which are both downstream targets of mTOR, in cells that had been serum deprived for 24 h. Indeed, a marked increase in the level of phosphorylation of 4E-BP1 (on Ser65) and p70^{S6K} (on Thr389) was evident in the MDA-MB-435/β4 transfectants relative to either the mock transfectants or the parental cells (Fig. 4 A). Phosphorylation of Ser65 of 4E-BP1 has been shown to be critical for dissociation of 4E-BP from eIF-4E (Gingras et al., 2001a). The reduced expression of 4E-BP1 in the β4 transfectants compared with the mock transfectants that is apparent in Fig. 4 A may reflect the possibility that the 4E-BP Ab does not recognize the hyperphosphorylated form of the protein as well as it recognizes the hypophosphorylated form.

The involvement of eIF-4E in VEGF translation was confirmed by the expression of an antisense eIF-4E oligonucleotide in the MDA-MB-435/β4 transfectants. As shown in Fig. 4 B, expression of this oligonucleotide reduced the level of VEGF protein significantly. In contrast, expression of the full-length eIF-4E cDNA increased the VEGF protein level by approximately twofold. These results, together with the polysome analysis data (Fig. 3), indicate that α6β4 regulates VEGF expression by eIF-4E–mediated, cap-dependent translation.

To confirm the specificity of the α6β4 integrin in mTOR signaling, the effects of integrin-mediated clustering on 4E-BP1 phosphorylation were assessed. A substantial induction of Akt, 4E-BP1, and p70^{S6K} phosphorylation was observed upon α6β4 integrin clustering in the β4 transfectants but not in the mock transfectants (Fig. 4 C). In contrast, clustering of the α5β1 integrin did not stimulate phosphorylation of these molecules in either the mock or β4 transfectants. Collectively, these data demonstrate the preferential ability of the α6β4 integrin to regulate the mTOR signaling pathway and, more importantly, the phosphorylation of 4E-BP1.

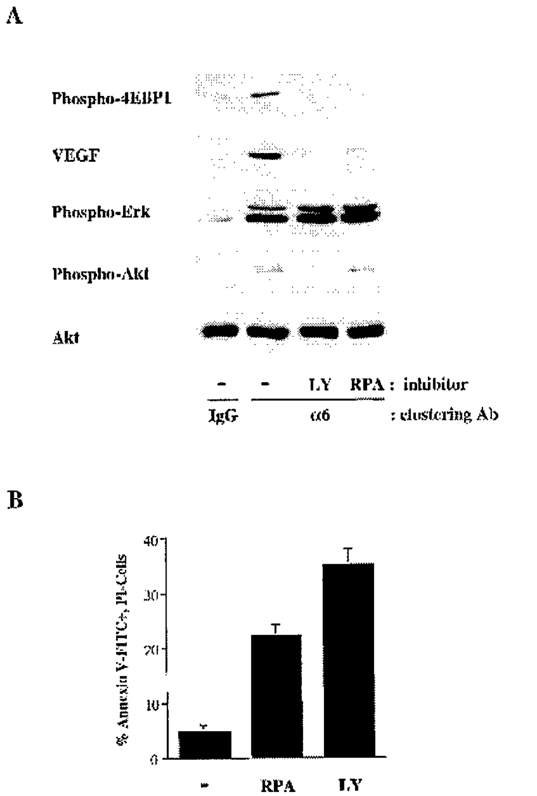

Figure 5. **Stimulation of 4E-BP1 phosphorylation, VEGF expression, and survival by the α6β4 integrin requires PI-3K and mTOR.** (A) MDA-MB-435 β4 transfectants (clone 3A7) were incubated with either DMSO (−), the PI-3K inhibitor LY 294002 (10 μM) (LY), or the mTOR-specific inhibitor rapamycin (50nM) (RPA) for 30 min and then incubated with either IgG or the α6 integrin antibody 2B7 as described in the legend to Fig. 2. Extracts of these cells were immunoblotted for phospho–4E-BP1 (Ser65), VEGF, phospho-Erk (recognizing phosphorylated isoforms of ERK1 and ERK2), phospho-Akt (Ser 473), and total Akt. Similar data were obtained in three experiments. (B) MDA-MB-435 β4 transfectants (clone 3A7) were maintained at low serum (0.5%) medium for 24 h in the presence of either rapamycin (50nM) (RPA), LY 294002 (10 μM) (LY), or DMSO (−). Apoptosis was assayed as described in the Materials and methods and is reported as the percentage of annexin V–FITC-positive, PI-negative cells. The data shown are mean values (± SD) of a representative experiment performed in triplicate.

To establish that PI-3K and mTOR are required for 4E-BP1 phosphorylation and VEGF expression, we performed the antibody clustering experiments in the presence of the PI-3K–specific inhibitor LY294002 and the mTOR-specific inhibitor rapamycin (Fig. 5). As shown in Fig. 5 A, both of these inhibitors blocked the α6β4-mediated induction of 4E-BP1 phosphorylation and VEGF expression. Although rapamycin did not block Akt phosphorylation, LY294002 did inhibit its phosphorylation, confirming that Akt acts upstream of mTOR and downstream of PI-3K (Fig. 5 A). These inhibitors did not block the phosphorylation of ERK1 and ERK2 (Fig. 5 A).

Finally, we investigated the importance of the mTOR pathway in survival, using rapamycin and LY294002. As shown in Fig. 5 B, rapamycin treatment increased the apoptosis of the MDA-MB-435/β4 transfectants fivefold and LY294002 treatment increased their apoptosis eightfold. These results indicate that the PI-3K–mTOR pathway is critical for the survival of these cells.

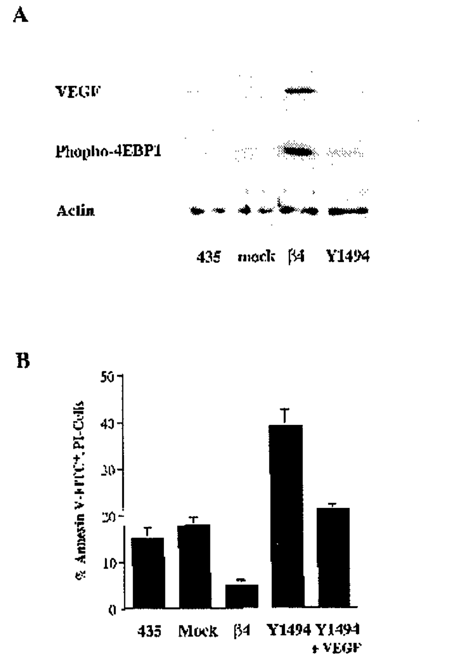

Figure 6. **Y1494 in the β4 cytoplasmic domain is required for α6β4 stimulation of 4E-BP1 phosphorylation, VEGF expression, and survival.** (A) MDA-MB-435 parental cells (435), mock transfectants (clone 6D7), wild-type β4 transfectants (clone 3A7), and Y1494F mutant transfectants (clone E1h) were maintained in low serum (0.5% FBS) for 24 h. Extracts from these cells were analyzed by immunoblotting to assess the relative expression of VEGF and 4E-BP1 phosphorylation. The relative amount of actin was also determined as a control for protein loading. Similar results were obtained in three experiments. (B) Aliquots of the same cell populations described in A were assayed for the level of apoptosis after a 24-h incubation in low serum (0.5% FBS) medium. Apoptosis was assayed as described in the Materials and methods and is reported as the percentage of annexin V–FITC-positive, PI-negative cells. The data shown are mean values (± SD) of three experiments performed in triplicate.

Identification of a specific tyrosine residue in the β4 cytoplasmic domain required for α6β4 stimulation of 4E-BP1 phosphorylation and VEGF expression

Recently, a critical tyrosine residue (Y1494) was identified in the third fibronectin type III repeat of the β4 cytoplasmic domain, and this tyrosine was shown to be essential for activation of PI-3K by α6β4 (Shaw, 2001). To assess the importance of Y1494 in 4E-BP1 phosphorylation and VEGF expression, stable subclones of MDA-MB-435 cells were generated that expressed α6β4 containing a Y1494F mutation. As shown in Fig. 6 A, VEGF protein expression was barely detectable in these transfectants compared with the wild-type transfectants. Also, the steady-state level of 4E-BP1 phosphorylation was substantially lower in the Y1494F mutant transfectants than in the wild-type β4 transfectants. Interestingly, these mutant transfectants also exhibited an

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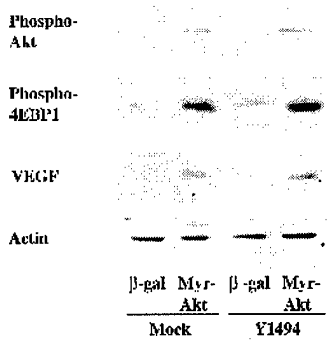

Figure 7. **Expression of a constitutively active Akt construct mimics the effects of α6β4 integrin expression and signaling.** MDA-MB-435 mock transfectants (clone 6D7) and Y1494F mutant transfectants (clone E1h) were infected with adenoviruses that expressed either β-galactosidase or Myr-Akt as described in the Materials and methods. Subsequently, the cells were incubated in low serum (0.5% FBS) medium for 24 h. Extracts of these cells were immunoblotted to assess the relative phosphorylation of Akt and 4E-BP1, as well as total expression of VEGF and actin.

eightfold higher level of apoptosis than the wild-type β4 transfectants in response to serum deprivation (Fig. 6 B). The apoptosis of the mutant cells was reduced substantially by the addition of recombinant VEGF (Fig. 6 B), a result that substantiates the importance of VEGF in the survival of these cells. Together, these findings highlight the importance of the β4 cytoplasmic domain and PI-3K signaling in the regulation of VEGF expression and tumor cell survival.

Expression of constitutively active Akt stimulates 4E-BP1 phosphorylation and VEGF expression in the absence of α6β4 signaling

The hypothesis that activation of Akt is a major determinant for the stimulation of 4E-BP1 phosphorylation and VEGF expression was assessed by expressing a constitutively active Akt construct in MDA-MB-435 cells that are deficient in α6β4 signaling. For this purpose, we used MDA-MB-435/mock transfectants that lack α6β4 expression and the MDA-MB-435/β4 Y1494F transfectants, described above, which are deficient in α6β4-mediated activation of PI-3K. These cells were infected with adenoviruses that encoded either a myristoylated Akt (Myr-Akt) construct or β-galactosidase as a control. As shown in Fig. 7, expression of Myr-Akt stimulated 4E-BP1 phosphorylation and VEGF expression substantially in both populations of transfectants in comparison to cells that expressed β-galactosidase. This result indicates the critical importance of Akt activation by α6β4 for stimulating VEGF expression.

α6β4 regulates 4E-BP1 phosphorylation, VEGF expression, and survival in carcinoma cells that express this integrin endogenously

Given that the data reported above are based on the exogenous expression of α6β4 in α6β4-deficient carcinoma cells, we sought to extend our findings to cells that express this integrin endogenously, a pattern that is typical of most carcinoma cells. For this purpose, we used MDA-MB-231 breast carcinoma cells because they express the α6β4 and α5β1 integrins (Plopper et al., 1998; Mukhopadhyay et al., 1999; Saad et al., 2000). Initially, we confirmed that the survival of these cells is dependent on their expression of VEGF. As shown in Fig. 8 A, expression of a VEGF antisense oligonucleotide in these cells (Bachelder et al., 2001) resulted in an approximate fourfold increase in annexin V staining upon serum starvation compared with either untreated cells or cells that expressed the sense oligonucleotide.

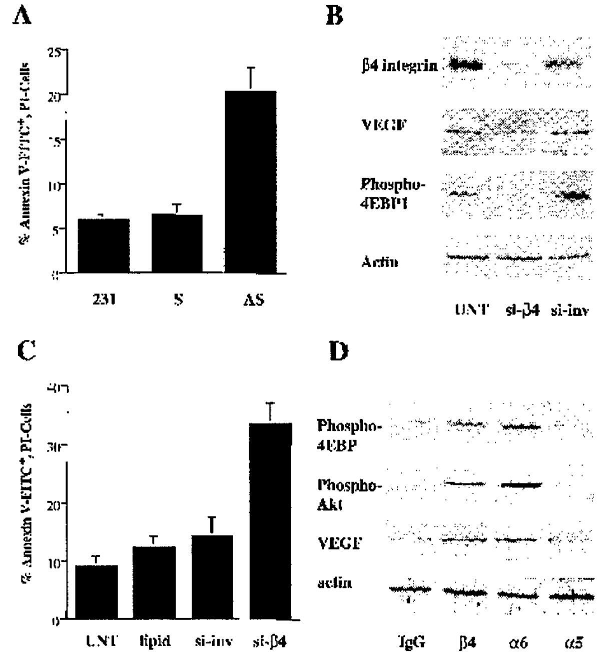

Figure 8. **α6β4 regulates 4E-BP1 phosphorylation, VEGF expression, and survival in carcinoma cells that express this integrin endogenously.** (A) Parental MDA-MB-231 cells and cells transfected with antisense or sense VEGF oligonucleotides were maintained in low serum (0.5% FBS) medium for 24 h. Apoptosis was assayed as described in the Materials and methods and is reported as the percentage of annexin V–FITC-positive, PI-negative cells. The data shown are mean values (± SD) of two separate experiments performed in triplicate. (B) MDA-MB-231 cells were left untreated (UNT) or were transfected with either an RNAi specific for the β4 integrin (si-β4) or the corresponding inverted sequence (si-inv). After 72 h, the cells were placed in medium containing low serum (0.5% FBS) for an additional 24 h and then extracted. Extracts of these cells were immunoblotted as described in the legend to Fig. 4 to assess expression of β4 integrin, VEGF, and actin, as well as the phosphorylation of 4E-BP1. Similar results were observed in three independent trials. (C) Apoptosis was assessed in the same populations of cells and is reported as the percentage of annexin V–FITC-positive, PI-negative cells. The data shown are mean values (± SD) of three independent experiments performed in triplicate. (D) MDA-MB-231 cells were maintained in low serum (0.5% FBS) medium for 24 h and harvested by trypsin treatment. The suspended cells were incubated with integrin-specific antibodies (β4 integrin, A9; α6 integrin, 2B7; α5 integrin, Sam1) or IgG for 30 min in suspension and allowed to adhere on anti-IgG–coated plates for 30 min. Extracts of these cells were immunoblotted with phosphospecific antibodies to assess the relative phosphorylation of Akt and 4E-BP1, as well as with antibodies specific for VEGF and actin. Similar results were obtained in five experiments.

To establish a role for α6β4 in regulating VEGF expression and survival rigorously, we used a small interfering

RNA (RNAi) approach to inhibit β4 expression in MDA-231 cells. RNAis specific for the β4 subunit and the corresponding inverted sequence were designed and expressed in these cells by transfection. The cells were maintained in low serum (0.5%) for 24 h after transfection and then analyzed. As shown in Fig. 8 B, cells transfected with the RNAi specific for β4 exhibited a significant reduction in β4 expression in comparison with either untransfected cells or cells transfected with the inverted sequence. Importantly, the reduction in β4 expression by RNAi coincided with a marked reduction in 4E-BP1 phosphorylation and in the steady-state level of VEGF (Fig. 8 B), as well as an approximate threefold increase in annexin V staining (Fig. 8 C). These results link α6β4 expression directly to 4E-BP1 phosphorylation, VEGF expression, and survival in a carcinoma cell line that expresses endogenous α6β4.

Subsequently, we performed antibody clustering experiments to substantiate the regulation of VEGF expression by α6β4 (Fig. 8 D). Clustering of the α6β4 integrin with either an α6 integrin–specific antibody (mAb 2B7) or a β4 integrin–specific antibody (mAb A9) stimulated the phosphorylation of 4E-BP1 and Akt, and increased VEGF expression. In contrast, no induction of VEGF expression or stimulation of either 4E-BP1 or Akt phosphorylation was observed upon clustering with an α5 integrin–specific antibody (mAb Sam1) or IgG.

Discussion

This study establishes a novel mechanism by which integrins regulate growth factor expression. Specifically, our findings demonstrate the ability of a specific integrin (α6β4), which has been implicated in carcinoma progression (Mercurio and Rabinovitz, 2001), to stimulate the translation of VEGF and sustain a VEGF autocrine loop that is essential for survival. More specifically, we define a signaling pathway regulated by α6β4 that involves the preferential ability of this integrin to stimulate the phosphorylation of 4E-BP1 by activating the PI-3K–Akt pathway. As shown previously, this phosphorylation event dissociates 4E-BP1 from eIF-4E, enabling this key elongation factor to mediate the translation of VEGF and other functionally important molecules (De Benedetti and Harris, 1999; Gingras et al., 1999, 2001b; McKendrick et al., 1999). Moreover, the polysome analysis and antisense eIF-4E results we provide indicate that α6β4 stimulation of VEGF translation is cap dependent and probably doesn't involve the internal ribosome entry sites that are present in the VEGF mRNA (Huez et al., 1998; van der Velden and Thomas, 1999). Our data extend earlier reports on the involvement of eIF-4E, VEGF, and α6β4 in carcinoma progression by linking these molecules in a common signaling pathway that promotes tumor survival. Furthermore, they reveal a role for integrins in regulating growth factor expression by stimulating protein translation.

An important and novel aspect of our findings is that they add a new dimension to the understanding of how integrins promote cell survival. The widely accepted notion is that integrins, often in concert with growth factor receptors, activate specific signaling pathways that sustain survival (Taylor et al., 1999; Liu et al., 2000). We demonstrate here that the survival function of integrins may not only be mediated by the activation of a key survival kinase such as Akt and the consequent effects of Akt on apoptotic signaling (Datta et al., 1999) but also by the Akt-dependent translation and expression of growth factors, such as VEGF, that promote survival in an autocrine, and possibly paracrine, fashion. In other terms, our results reveal that VEGF is a novel target of Akt signaling by integrins that is important for survival and distinct from known survival factors that are downstream of Akt, such as Bad (Datta et al., 1999). Importantly, our recent observation that VEGF stimulates the PI-3K–Akt pathway in carcinoma cells (Bachelder et al., 2001), in conjunction with our finding that α6β4 signaling enhances VEGF expression, leads to the conclusion that integrin-mediated activation of PI-3K–Akt is amplified by integrin-stimulated VEGF expression. Moreover, we show that this amplification of PI-3K–Akt activity is important for carcinoma survival.

Although α6β4 activates PI-3K in carcinoma cells (Gambaletta et al., 2000; Nguyen et al., 2000; Hintermann et al., 2001; Trusolino et al., 2001), no attempt had been made to link this signaling event with downstream effectors that regulate protein translation, namely mTOR and 4E-BP1. One reason that this possibility had not been explored is because a role for α6β4 in regulating either protein translation or growth factor expression was not obvious. In fact, almost all of the functional studies on α6β4 in carcinoma cells have focused on its role in promoting migration and invasion, and on the mechanism by which α6β4-mediated signaling influences these processes (Mercurio, 1990; Shaw et al., 1997; Gambaletta et al., 2000; Trusolino et al., 2001). Our motivation to examine a possible connection between α6β4 and VEGF translation was based on our interest in understanding the mechanisms by which these molecules promote the survival of carcinoma cells. Indeed, our results establish a role for α6β4 in survival signaling by regulating VEGF translation, but the implications of these findings are more widespread. For example, recent studies that have argued that α6β4 is necessary for growth factor receptor (erbB2, c-met) activation of PI-3K (Gambaletta et al., 2000; Trusolino et al., 2001) raise the interesting possibility of an intimate functional association among specific growth factor receptors, α6β4, VEGF, and PI-3K, all of which have been implicated in tumor progression.

Surprisingly, few studies have examined the role of integrin signaling in regulating protein translation (e.g., Pabla et al., 1999). Indeed, there has been much more interest in defining the contribution of integrins to transcription. The ability of integrins to regulate translation, however, provides a mechanism for altering cell function rapidly, by increasing the expression of specific proteins. This possibility is exemplified by our finding that ligation of the α6β4 integrin resulted in a significant increase in VEGF protein within 60 min (Fig. 2 C). Given the fact that eIF-4E is rate limiting for the translation of proteins involved in growth control and other critical cell functions (De Benedetti and Harris, 1999), the hypothesis can be formulated that integrin-mediated regulation of translation contributes to the ability of cells to alter their behavior rapidly in response to changes in their microenvironment. This hypothesis is particularly relevant to our interest in the regulation of VEGF expression. Al-

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though much of the work in this area has focused on the ability of hypoxia to stimulate VEGF transcription and increase the stability of VEGF mRNA (von Marschall et al., 2001), it has become apparent that translational control is also important (Kevil et al., 1996; De Benedetti and Harris, 1999). Moreover, our recent finding that VEGF is essential for the survival of breast carcinoma cells in normoxia substantiates the functional importance of integrin-mediated regulation of VEGF expression (Bachelder et al., 2001).

The fact that our data implicate eIF-4E in tumor cell survival is of considerable interest because recent studies have revealed an important role for this elongation factor in cancer (DeFatta et al., 1999, 2000; Ernst-Stecken, 2000; Berkel et al., 2001). Overexpression of this factor in NIH3T3 cells, as well as other "normal" cells, stimulates division and can induce their transformation (Fukuchi-Shimogori et al., 1997). These findings are consistent with the reports that the expression of eIF-4E is elevated in solid tumors compared with normal tissue (De Benedetti and Harris, 1999). Moreover, hypoxia, a pathophysiological stress that provides a selective pressure for the survival of aggressive tumor cells, enhances eIF-4E expression (DeFatta et al., 1999). Together, these observations highlight an important role for translational control in human cancer. This role is substantiated by the fact that eIF-4E controls the translation of not only VEGF but also other molecules that influence tumor growth and survival such as c-Myc, cyclin D1, and FGF-2 (De Benedetti and Harris, 1999). From our perspective, we are intrigued by the reports that the α6β4 integrin is associated with the progression of many solid tumors, and its expression has been correlated with a poorer prognosis in patients with some of these tumors (Mercurio and Rabinovitz, 2001). Our finding that α6β4 can induce the translational function of eIF-4E by regulating the phosphorylation of 4E-BP1 provides one mechanism to account for the role of this integrin in cancer.

Materials and methods

Cells

MDA-MB-231 and MDA-MB-435 breast carcinoma cells were obtained from the Lombardi Breast Cancer Depository at Georgetown University. They were grown in low glucose DME containing 10% FBS, 1% penicillin–streptomycin, and 25 mM Hepes. For inhibitor experiments, cells were harvested by trypsinization and suspended cells were incubated with rapamycin (Calbiochem) at 100 nM or LY 294002 (Calbiochem) at 10 μM on ice for 30 min before they were plated at 37°C for the experiment.

The generation of MDA-MB-435 subclones expressing the α6β4 integrin has been described previously (Shaw et al., 1997). Tyrosine residue 1494 in the β4 subunit was mutated to a phenylalanine residue using the Quickchange site-directed mutagenesis kit (Stratagene), and stable subclones of MDA-MB-435 cells that expressed α6β4 containing this mutant β4 subunit were generated (Shaw, 2001).

For adenoviral infection, cells were grown in DME containing 10% FBS until they reached 50% confluency. At this point, the culture medium was changed to DME containing 0.5% FBS. Viral dilutions were prepared from purified viral stocks in DME containing 0.5% FBS and the cells were infected for 4 h. At the end of the infection period, the virus-containing medium was removed and the cells were washed once with PBS, and incubated for an additional 12 h in DME containing 10% FBS.

Apoptosis assays

To induce apoptosis, cells were incubated in DME containing 0.5% FBS for 24 h at 37°C. Subsequently, both adherent and nonadherent cells were harvested and their level of apoptosis was assessed using annexin V–FITC. In brief, cells were washed once with serum-containing medium, once with PBS, once with annexin V–FITC buffer (10 mM Hepes-NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), and then incubated for 15 min at room temperature with 5 μg/ml annexin V–FITC (Biosource International). After washing once with annexin V buffer, the samples were resuspended in the same buffer and analyzed by flow cytometry. Immediately before the analysis, 5 μg/ml propidium iodide (PI) (Biosource International) was added to distinguish apoptotic cells from necrotic cells.

Quantitative real-time PCR

Quantitative analysis of VEGF mRNA expression was performed by real-time PCR using an ABI Prism 7700 sequence detection system (PerkinElmer) and SYBR green master mix kit as described previously (Bachelder et al., 2001). Sequences of primers and probes were as follows: VEGF forward primer, 5′-GAAGTGGTGAAGTTCATGGATGTCTA-3′; VEGF reverse primer, 5′-TGGAAGATGTCCACCAGGGT-3′; VEGF probe, 5′-/TET/AGCGCAGCTACTGCCATCCAATCG/TAM/-3′; β-actin forward primer, 5′-TCACCATGGATGATGATATCGC-3′; β-actin reverse primer, 5′-AAGCCGGCCTTGCACAT-3′; and β-actin probe, 5′-/FAM/CGCTCGTCGTCGACAACGGCT/TAM/-3′.The data obtained are presented as the mean ratio of VEGF to β-actin mRNA (± SD) obtained from triplicate samples.

VEGF antisense oligonucleotide experiments

A VEGF antisense 2′-*O*-methyl phosphorothioate oligodeoxynucleotide (5′-CACCCAAGACAGCAGAA-3′) and a sense 2′-*O*-methyl phosphorothioate oligodeoxynucleotide (5′-CTTTCTGCTGTCTTGGGTG) (provided by Greg Robinson, Children's Hospital, Boston, MA) were used to transfect MDA-MB-435 β4 transfectants at a concentration of 0.3 μM in the presence of lipofectin reagent (2 μg/ml; GIBCO BRL). The experimental details for this approach have been described previously (Bachelder et al., 2001). In addition, the same protocol was used to express antisense and sense eIF-4E oligonucleotides, which were gifts from Arigo De Benedetti (Louisiana State University, Shreveport, LA) (DeFatta et al., 2000).

RNAi experiments

An RNAi specific for the β4 integrin subunit (GAGCUGCACGGAGUGUGUC) as well as the inverted sequence (CUGUGUGAGGCACGUCGAG) were designed and synthesized by Dharmacon, Inc. MDA-231 cells at 30% confluency were transfected with 300 pmoles of RNAi using TKO lipids (Mirus). Subsequently, the cells were maintained in complete medium for 72 h and in medium containing 0.5% FBS for an additional 24 h before analysis.

Polysome analysis

Cells (3×10^7) were maintained in medium containing low serum (0.5% FBS) for 24 h and then pretreated with 100 μg/ml cycloheximide (Calbiochem) for 15 min at 37°C before being harvested. After washing once with PBS containing 100 μg/ml cycloheximide, the cells were resuspended in 0.5 ml of a modified U+S buffer (Davies and Abe, 1995). This buffer was composed of 200 mM Tris-HCl (pH 8.8), 25 mM MgCl2, 5 mM EGTA (pH 8.0), 150 mM KCl, 10 μg/ml heparin, 5 mM DTT, 1% sodium deoxycholate, 2% polyoxyethylene 10-tridecy ester, 100 μg/ml cycloheximide, and 200 mM sucrose. Ribonuclease inhibitor (Amersham Biosciences) was added to a final concentration of 0.5 U/μl. Cells were homogenized with 20–25 strokes in a Kontes tissue homogenizer, followed by centrifugation for 5 min at 14,000 *g*. The supernatant was collected and frozen at −80°C until further use. Sucrose gradients (15–50%, wt/wt) were layered with 300 μl of cleared cell extract, which was then centrifuged at 160,000 *g* for 2 h. Fractions (0.75–0.375 ml) were withdrawn from the top of the gradient and monitored for absorbency at 254 nm using an ISCO syringe pump with UV-6 detector. Total RNA from the sucrose gradient fractions was extracted using Trizol LS (Life Technologies) according to the manufacturer's instruction. Quantitative real-time PCR was used to measure the VEGF mRNA level in each fraction as described above.

Integrin signaling experiments

Cells were harvested by trypsin treatment and washed twice with DME containing 25 mM Hepes and 0.1% BSA. After washing, the cells were resuspended in the same buffer at a concentration of 2×10^6 cells/ml and incubated for 30 min with integrin-specific antibodies (4 μg/ml) or with either mouse or rat IgG (4 μg/ml). The cells were washed once, resuspended in the same buffer, and added to plates that had been coated overnight with either the anti–mouse or rat IgG. After a 60-min incubation at 37°C, the cells that had attached to integrin-specific antibodies were washed twice with cold PBS and solubilized at 4°C for 10 min using RIPA buffer (20 mM Tris buffer, pH 7.4, containing 0.14 M NaCl, 1% NP-40, 10% glycerol, 1 mM sodium orthovanadate, 2 mM PMSF, 5 μg/ml aprotinin,

pepstatin, and leupeptin). The IgG-treated cells were harvested by centrifugation and solubilized with RIPA buffer.

Protein analysis

Aliquots of cell extracts containing equivalent amounts of protein were solubilized using 5× sample buffer containing 100 mM DTT and then incubated at 100°C for 15 min. These extracts were resolved by SDS-PAGE and transferred to nitrocellulose filters. The filters were blocked for 1 h using a 50 mM Tris buffer, pH 7.5, containing 0.15 M NaCl, 0.05% Tween-20 (TBST), and 5% (wt/vol) Carnation dry milk. The filters were incubated overnight in the same buffer containing antibodies specific for p70S6K, 4EBP antibodies (Santa Cruz Biotechnology, Inc.), actin (ICN Biomedicals), and VEGF (clone 618, provided by Donald Senger, Beth Israel Deaconess Medical Center). After three, 10-min washes in TBST, the filters were incubated for 1 h in blocking buffer containing HRP-conjugated secondary antibodies. After three 10-min washes in TBST, proteins were detected by ECL (Pierce Chemical Co.).

For immunoblots involving phosphospecific antibodies, the filters were blocked for 1 h using a 10 mM Tris buffer, pH 7.5, containing 0.5 M NaCl, 0.1% Tween-20, and 2% (wt/vol) BSA. The filters were washed briefly and then incubated overnight at 4°C in the same blocking buffer containing antibodies specific for phospho-p70S6K (Thr-389; Cell Signaling Technology), phospho-4E-BP1 (Ser-65; Cell Signaling Technology), phospho-Erk (E10; Cell Signaling Technology), and phospho-Akt (Ser-473 clone 4E2; Cell Signaling Technology). After washing, the filters were incubated for 1 h in blocking buffer containing HRP-conjugated secondary antibody and the proteins were detected by ECL.

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[CANCER RESEARCH 61, 5736–5740, August 1, 2001]

Advances in Brief

Vascular Endothelial Growth Factor Is an Autocrine Survival Factor for Neuropilin-expressing Breast Carcinoma Cells[1]

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Abstract

We identify a novel function for the vascular endothelial growth factor (VEGF) in its ability to stimulate an autocrine signaling pathway in metastatic breast carcinoma cells that is essential for their survival. Suppression of VEGF expression in metastatic cells *in vitro* induced their apoptosis, in addition to inhibiting the constitutively elevated phosphatidylinositol 3′-kinase activity that is characteristic of these cells and important for their survival. Hypoxia enhanced the survival of metastatic cells by increasing VEGF expression. The importance of the VEGF receptor neuropilin was indicated by the ability of a neuropilin-binding VEGF isoform to enhance breast carcinoma survival. Moreover, the expression of neuropilin in neuropilin-deficient breast carcinoma cells protected them from apoptosis. The identification of this VEGF autocrine signaling pathway has important implications for tumor metastasis and therapeutic intervention.

Introduction

Breast epithelial cells are able to survive and differentiate because of the tissue architecture and growth factor milieu present in the mammary gland (1). This rich environment, however, is progressively lost during transformation, especially as malignant cells become invasive and metastatic. One of the most formidable barriers to tumor survival is hypoxia (2). Although hypoxia kills most normal cells and some tumor cells (3–5), it also provides a strong selective pressure for the survival of the most aggressive and metastatic cells (6). These considerations substantiate the importance of defining the molecular characteristics of tumor cells that enable their survival. One survival strategy utilized by breast carcinomas and other tumors is the secretion of proteins that elicit an angiogenic response. For example, vascular permeability factor or VEGF[5] appears to be an essential factor for breast carcinoma progression (7–9). It is widely assumed that the function of VEGF produced by breast carcinoma and tumor stromal cells is to stimulate angiogenesis by acting in a paracrine fashion on vicinal endothelium (8, 10). Surprisingly, however, the possibility that VEGF functions in an autocrine fashion on breast carcinoma cells to stimulate signaling pathways that maintain their survival has not been considered. This autocrine activity of VEGF could be important for survival in hypoxic, poorly vascularized areas of solid tumors. Our results define a novel signaling pathway in breast carcinoma cells involving VEGF, the VEGF receptor neuropilin, and PI3-kinase that is likely to play an important role in breast carcinoma progression.

Materials and Methods

Cells. MDA-MB-231, MDA-MB-435, and MDA-MB-453 cells were obtained from the Lombardi Breast Cancer Depository (Georgetown University). Primary endothelial cells were provided by Dr. Donald Senger (Beth Israel Deaconess Medical Center). For hypoxia experiments, cells were cultured in low-serum culture medium (DMEM/0.5% FBS) and maintained in either normoxic (5% CO_2, 20% O_2, 75% N_2) or hypoxic (5% CO_2, 3% O_2, 94.5% N_2) conditions for the indicated amounts of time.

VEGF Antisense Strategy. Cells (2×10^5 cells/well of a 12-well plate; 50% confluence) were transfected with either a VEGF antisense 2′-*O*-methyl phosphorothioate oligodeoxynucleotide (5′-CACCCAAGACAGCAGAAG-3′) or a VEGF sense 2′-*O*-methyl phosphorothioate oligodeoxynucleotide (5′-CTTTCTGCTGTCTTGGGTG) at a concentration of 0.3 μM in the presence of Lipofectin reagent (Life Technologies, Inc.; 2 μg/ml). These oligonucleotides contain 2′-*O*-methyl modifications at the last five nucleotides (3′). The design of these oligonucleotides has been described previously (11). After 4 h, the cells were washed with PBS and allowed to recover in DMEM/10% FBS. These conditions were determined to be optimal for inhibiting VEGF expression in these cell lines as determined by quantitative RT-PCR (data not shown).

Quantifying VEGF mRNA. mRNA was isolated from cellular extracts using the RNEasy kit (Qiagen). cDNA was synthesized from this RNA using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) and quantified by RT-PCR. Primers and probes were synthesized by Oligo Therapeutics (Wilsonville, OR) and Perkin Elmer (Foster City, CA), respectively. Primer and probe sequences were analyzed for specificity of gene detection using the NCBI Blast module by first derivative primer melting curve software supplied by Perkin Elmer/Applied BioSystems. Quantitative analysis of gene expression was generated using an ABI Prism 7700 Sequence Detection System (TaqMan) and the SYBR Green master mix kit. The sequences of the PCR primer pairs (5′ to 3′) that were used for each gene are as follows: VEGF forward, 5′-GGAGATCCTTCGAGGAGCACTT-3′; VEGF reverse, 5′-GGCGATTTAGCAGCAGATATAAGAA-3′; cyclophilin forward, 5′-CAGACGCCACTGTCGCTTT-3′; and cyclophilin reverse, 5′-TGTCTTTGGAACTTTGTCTGCAA-3′.

Apoptosis Assays. Apoptosis was assessed as described previously (12) using annexin V-FITC (Biosource) and PI (Biosource), annexin V-phycoerythrin (Promega), or the Apoptag kit (Oncor).

VEGF Receptor Expression. To assess VEGF receptor expression, proteins were extracted from cells in lysis buffer [20 mM Tris (pH 7.4), 0.14 M NaCl, 1% NP40, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 50 μg/ml leupeptin, and 1 mM sodium orthovanadate]. Cellular debris was removed from these extracts by centrifugation at 12,000 rpm for 10 min at 4°C, and the concentration of total cellular protein was determined in these samples using the Biorad reagent (Biorad). Equivalent amounts of total cellular protein were subjected to reducing SDS-PAGE (6%), transferred to nitrocellulose, and probed with mouse monoclonal antibodies specific for either neuropilin or KDR, followed by HRP-conjugated goat

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[5] The abbreviations used are: VEGF, vascular endothelial growth factor; PI3-kinase, phosphatidylinositol 3′-kinase; RT-PCR, real-time PCR; PI, propidium iodide; PKB, protein kinase B; HRP, horseradish peroxidase.

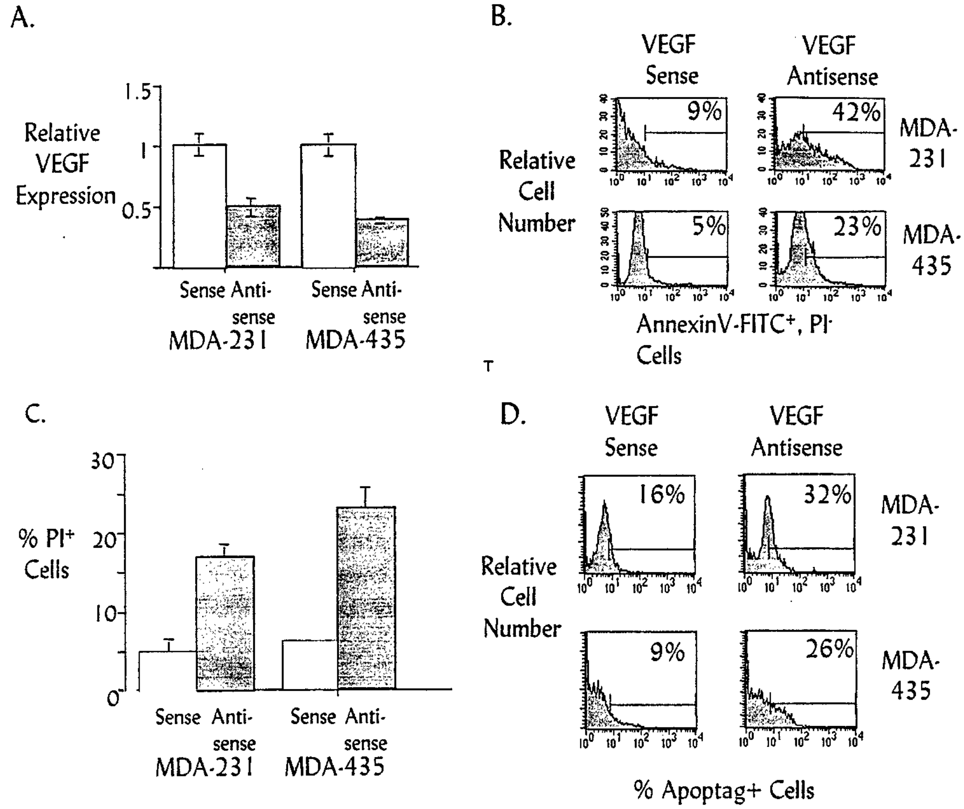

Fig. 1. Decreasing VEGF expression in metastatic breast carcinoma cells promotes their apoptosis. MDA-MB-231 and MDA-MB-435 cells were transfected with either a VEGF antisense or a control sense oligonucleotide and harvested 15 h (MDA-231) or 40 h (MDA-435) after transfection. *A*, the amount of VEGF mRNA in extracts from these cells was quantified by RT-PCR. The data are presented as the mean VEGF:cyclophilin mRNA ratio (±SD) obtained from triplicate samples. *B*, the level of apoptosis is indicated as the percentage of annexin$^+$, PI$^-$ cells. Similar results were obtained in five separate trials. *C*, the percentage of PI$^+$ cells (±SD) is indicated. Similar results were obtained in three independent experiments. *D*, the level of apoptosis in these transfectants was measured by performing terminal deoxynucleotidyl transferase-mediated nick end labeling-based reactions using the Apoptag kit (Oncor). The data are presented as the percentage of Apoptag-positive cells.

antimouse IgG. These receptors were then detected by enhanced chemiluminescence (Pierce).

Results and Discussion

The Survival of Metastatic Breast Carcinoma Cells *in Vitro* Is Dependent on VEGF. To assess the importance of VEGF in breast carcinoma survival, we examined the effects of reducing VEGF expression on the survival of two well-characterized metastatic cell lines (MDA-MB-231 and MDA-MB-435). Both of these cell lines express VEGF (13) and exhibit spontaneous metastasis to lungs upon orthotopic injection in the mammary fat pad (14). These cells were transfected with VEGF antisense or sense oligonucleotides and allowed to recover in culture medium containing 10% FBS. As shown in Fig. 1*A*, expression of the antisense oligonucleotide in these cells reduced the steady-state levels of VEGF mRNA by approximately 50% compared with the sense oligonucleotide, as measured by quantitative RT-PCR. The effect of decreased VEGF expression on cell survival was then assessed by incubating these cells with annexin V-FITC and PI. A 4-fold increase in annexin V binding was observed in antisense-transfected cells relative to both sense-transfected (Fig. 1*B*) and parental cells (data not shown). Also, a 3-fold increase in cell death in antisense-transfected cells relative to sense-transfected cells was observed, as determined by PI staining (Fig. 1*C*). Finally, we confirmed that these VEGF antisense-transfected cells were apoptotic using a terminal deoxynucleotidyl transferase-mediated nick end labeling-based assay (Apoptag) (Fig. 1*D*). Our ability to induce apoptosis in these metastatic cells by inhibiting VEGF expression, even in the presence of serum, indicates an essential function for this cytokine in their survival. The contribution of VEGF to tumor progression has been attributed exclusively to its angiogenic function. Our findings suggest that VEGF can also sustain breast carcinoma survival independently of angiogenesis by stimulating autocrine survival signaling in these cells.

VEGF Promotes Breast Carcinoma Survival by Stimulating the PI3-kinase Pathway. MDA-MB-231 cells exhibit constitutively elevated PI3-kinase activity (15).[6] Based on data in Fig. 1 and reports that VEGF stimulates PI3-kinase in endothelial cells (16–18), we hypothesized that VEGF maintains constitutively elevated PI3-kinase activity in MDA-MB-231 breast carcinoma cells and that this signaling is critical for their survival. To address this hypothesis, we assessed the effect of reducing VEGF expression in MDA-MB-231 cells on PI-3-kinase activity. Cells were transfected with antisense or sense oligonucleotides, and PI3-kinase activity was measured 15 h after transfection. As shown in Fig. 2*A*, a significant reduction in PI3-kinase activity was detected in antisense-transfected cells relative to sense-transfected cells. Densitometry of chromatograms from three experiments indicated that PI3-kinase activity was reduced by an average of 49% (±7.6% SD). Importantly, PI3-kinase activity was restored in antisense-transfected cells by the addition of exogenous VEGF (Fig. 2*A*). As a control, we observed that mitogen-activated protein kinase activity was not decreased in antisense-transfected cells (data not shown). These results demonstrate that the elevated level of PI3-kinase activity in MDA-MB-231 cells is the result of VEGF autocrine signaling. We also observed that treatment of these cells with the PI3-kinase inhibitor LY294002 (100 μM) induced their apoptosis, as indicated by a 2-fold increase in annexin-V-FITC-positive cells, as well as a 3.6-fold increase in PI-positive cells (Fig. 2*B*). These data indicate the importance of the VEGF-dependent activation of PI3-kinase in MDA-MB-231 cell survival.

Hypoxia Promotes Carcinoma Cell Survival by Increasing VEGF Expression. Given that VEGF is important for the survival of MDA-MB-231 cells in normoxia (Fig. 1), we predicted that hypoxia would provide a survival advantage for these cells by increasing VEGF expression. In addition to increasing their level of VEGF

[6] L. Shaw, unpublished data.

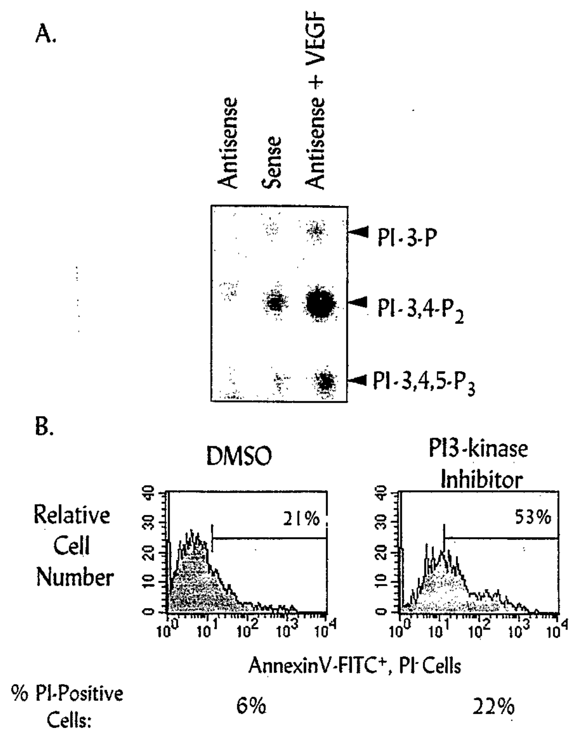

Fig. 2. VEGF is responsible for the constitutive PI3-kinase activity in MDA-MB-231 cells that is important for their survival. *A*, MDA-MB-231 cells were transfected with either the VEGF sense or antisense oligonucleotides and recovered in complete medium containing either no exogenous VEGF or recombinant $VEGF_{165}$ (100 ng/ml). PI3-kinase activity was assessed in extracts from these cells 15 h after transfection using methods described previously (22). The phosphorylated lipid products were resolved by thin layer chromatography as shown in this figure. The D3-phosphoinositides are indicated at the *right*. The relative amounts of radiolabeled PtdIns-3,4,5-P_3 were determined by densitometry (see "Results and Discussion"). *B*, MDA-MB-231 cells were incubated with DMSO (1:2500 dilution) or LY294002 (100 μM) in low-serum (0.5% FBS) culture medium for 48 h, and new drug-containing medium was added after 24 h. The data are presented as the mean percentage of cells from triplicate samples that were both annexin V-FITC positive and PI negative. The percentage of PI-positive cells is also indicated. Similar results were obtained in three trials.

expression (Fig. 3*A*), the exposure of serum-deprived MDA-MB-231 cells to hypoxia reduced their level of apoptosis significantly (Fig. 3*B*). Importantly, using the VEGF antisense oligonucleotide, we also demonstrated that the enhanced survival of these cells in hypoxia is VEGF dependent (Fig. 3*C*). Based on our observation that VEGF stimulates the PI3-kinase pathway in these cells in an autocrine manner (Fig. 2), we predicted that hypoxia would increase the activity of the serine/threonine kinase Akt/PKB, a downstream target of PI3-kinase, in these cells. The exposure of MDA-MB-231 cells to hypoxia significantly enhanced their level of Akt/PKB activity, as assessed by immunoblotting extracts from these cells with an antiserum specific for Akt/PKB molecules phosphorylated on serine residue 473 (Fig. 3*D*). These results provide evidence that hypoxia, a toxic stimulus, actually enhances the survival of breast carcinoma cells *in vitro* by augmenting VEGF expression and autocrine signaling.

Evidence for the Involvement of Neuropilin in VEGF Survival Function. In addition to the classical VEGF receptors KDR and Flt-1, neuropilin is a receptor that promotes VEGF function in endothelial cells (19). Interestingly, neuropilin expression in tumor cells may facilitate an angiogenic response by a mechanism that remains to be elucidated (20). Previous studies have indicated by Northern blotting that MDA-MB-231 (19, 21) and MDA-MB-435 (19) cells express neuropilin but not KDR. Based on these findings, we hypothesized that this novel VEGF receptor supports the autocrine survival function of VEGF in these breast carcinoma cells. First, we confirmed by immunoblotting that MDA-MB-231 and MDA-MB-435 cells express neuropilin but not KDR at the protein level (Fig. 4*A*). As a control, we demonstrated the presence of neuropilin and KDR proteins in an endothelial cell lysate by immunoblotting with these same antibodies (Fig. 4*A*).

To address the hypothesis that neuropilin promotes the VEGF-dependent survival of breast carcinoma cells, we compared the relative ability of VEGF splice variants that differ in receptor specificity to promote the survival of MDA-MB-231 cells. Specifically, we used $VEGF_{165}$, which binds to all of the three known VEGF receptors, and $VEGF_{121}$, which binds to KDR and Flt-1 but not to neuropilin (19).

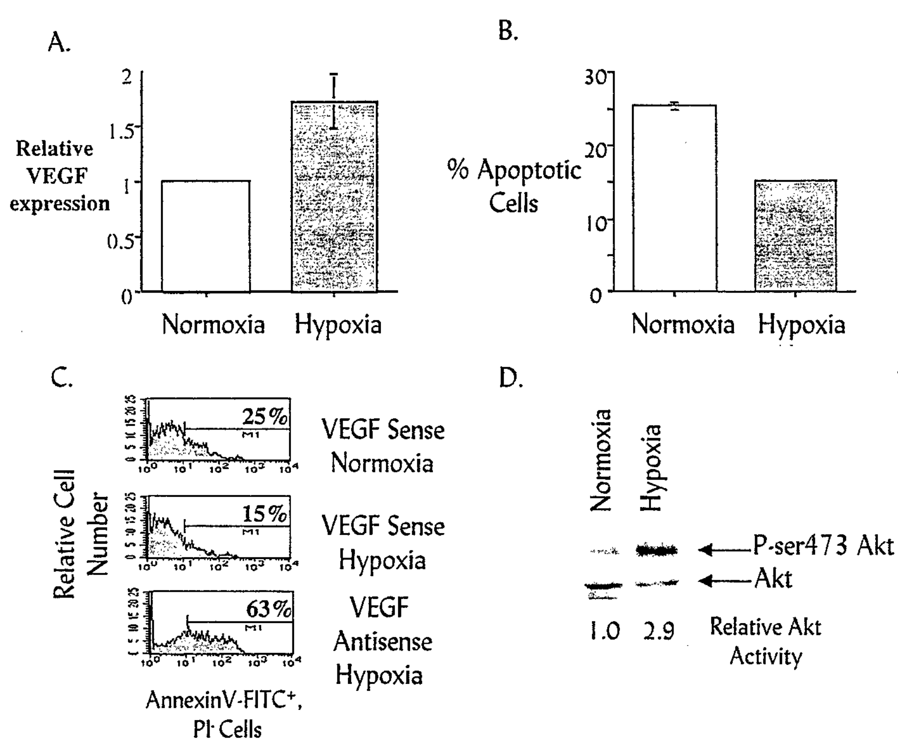

Fig. 3. Hypoxia enhances the survival of MDA-MB-231 cells by increasing VEGF expression. MDA-MB-231 cells were maintained in low-serum (0.5% FBS) culture medium in either normoxic or hypoxic conditions. *A*, after 48 h, VEGF mRNA levels were quantified by RT-PCR. The data are presented as the mean VEGF:cyclophilin mRNA ratio (±SD) obtained from triplicate samples. *B*, the level of apoptosis after 48 h is indicated as the mean percentage of cells from triplicate wells that were annexin$^+$, PI$^-$ (% *Apoptotic Cells*). Similar results were obtained in two additional trials. *C*, MDA-MB-231 cells were transfected with either the VEGF antisense or sense oligonucleotides. Fifteen h after transfection, these cells were cultured in either normoxia or hypoxia in low-serum (0.5% FBS) culture medium for an additional 48 h. The percentage of annexin V$^+$, PI$^-$ cells for each sample is indicated. Similar results were obtained in three independent experiments. *D*, after 6 h, Akt/PKB activity was assessed in extracts from these cells using methods described previously (12). Relative Akt activity (ratio of serine 473 phosphorylated to total cellular Akt) was determined by densitometry. Similar results were obtained in two additional trials.

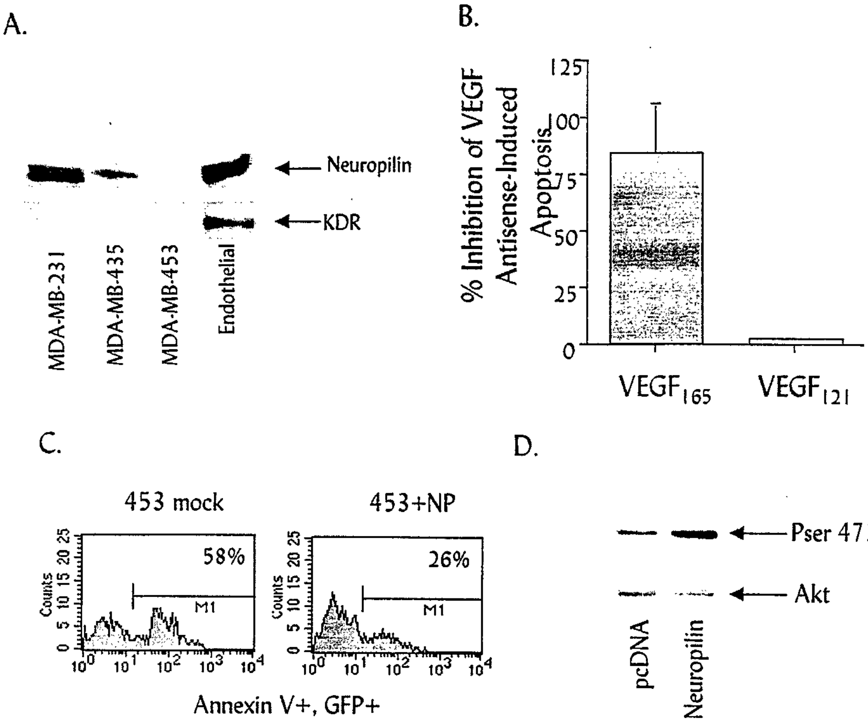

Fig. 4. Breast carcinoma cell expression of VEGF receptors. *A*, the expression of neuropilin and KDR in MDA-MB-231, MDA-MB-435, MDA-MB-453, and primary human endothelial cells was assessed by immunoblotting extracts from these cells with rabbit antisera specific for KDR (Sigma) or a monoclonal antibody specific for neuropilin (Santa Cruz), followed by HRP-conjugated goat antirabbit or antimouse IgG, respectively. KDR and neuropilin were visualized by enhanced chemiluminescence. *B*, MDA-MB-231 cells that had been transfected with the antisense and sense oligonucleotides were incubated in low-glucose DMEM containing heparin (1 μg/ml) and either no VEGF, recombinant $VEGF_{165}$ (R&D Systems; 100 ng/ml final concentration), or recombinant $VEGF_{121}$ (R&D Systems; 100 ng/ml final concentration). After 15 h, these cells were harvested, and the level of apoptosis was determined by staining with annexin-V-FITC and PI. The data are reported as the mean percent inhibition from three samples of VEGF antisense-induced apoptosis. Similar results were obtained in three trials. *C*, MDA-MB-453 cells (3×10^5 cells/well of a 6-well tissue culture plate) were transfected using the LipofectAMINE reagent (Life Technologies, Inc.) with either a myc-tagged plasmid encoding for full-length chicken neuropilin (provided by Dr. Jonathan Raper; 1 μg) or a control plasmid (1 μg), and a vector expressing green fluorescent protein (1 μg of pGFP). These cells were allowed to recover in complete culture medium for 12 h and then exposed to hypoxia in low-serum (0.5% FBS) medium for 48 h. The level of apoptosis in these cells was then assessed by annexin V-FITC and PI staining. The data represent the mean percentage of cells from three samples that were annexin positive and PI negative. Similar results were obtained in three separate trials. Importantly, neuropilin expression in these transfectants was confirmed by immunoblotting extracts from these cells with a HRP-conjugated antibody specific for the myc tag (data not shown). *D*, MDA-MB-453 cells were transfected with either a control plasmid or chicken neuropilin as described in (*C*). These cells were allowed to recover for twelve hours and then exposed to hypoxia in low-serum (0.5% FBS) medium for 48 h. Akt/PKB activity in these cells was assessed using previously described methods (12).

MDA-MB-231 cells were transfected with VEGF sense or antisense oligonucleotides and maintained in medium containing either no exogenous VEGF, $VEGF_{165}$, or $VEGF_{121}$. The level of apoptosis in these cells was then assessed by annexin V-FITC and PI staining. Importantly, the incubation of the antisense-transfected cells with $VEGF_{165}$, but not with $VEGF_{121}$, inhibited their apoptosis significantly (Fig. 4*B*). The ability of a neuropilin-binding splice variant of VEGF, but not a variant lacking neuropilin specificity, to augment the survival of MDA-MB-231 cells suggests the importance of neuropilin in VEGF autocrine signaling in these breast carcinoma cells.

Neuropilin has been detected in metastatic but not in nonmetastatic tumors (19). In agreement with this observation, we found that neuropilin is expressed in the metastatic cell lines MDA-MB-231 and MDA-MB-435, but not in the nonmetastatic cell line MDA-MB-453 (Fig. 4*A*). Based on these data and our results showing that a neuropilin-binding VEGF splice variant enhances breast carcinoma survival (Fig. 4*B*), we hypothesized that cells lacking neuropilin expression, such as the nonmetastatic breast carcinoma cell line MDA-MB-453, cannot support VEGF survival signaling. To test this prediction, we compared the relative abilities of mock-transfected and neuropilin-transfected MDA-MB-453 cells to survive in hypoxia. We observed that the exposure of mock-transfected MDA-MB-453 cells to hypoxia induced a significant level of apoptosis in these cells (Fig. 4*C*). In contrast, neuropilin-expressing MDA-MB-453 cells were protected from hypoxia-induced apoptosis (Fig. 4*C*). Finally, as evidence that neuropilin activates the PI3-kinase pathway in these cells, we observed that neuropilin-expressing MDA-MB-453 cells exhibit higher levels of Akt/PKB activity than do mock transfectants (Fig. 4*D*).

Studies on neuropilin function have highlighted its role in endothelial cells as a critical KDR coreceptor that facilitates VEGF-mediated signaling through this tyrosine kinase-linked receptor (19). Our studies are the first to identify a specific function for neuropilin in tumor cells, namely, its importance in maintaining breast carcinoma survival. In addition, these studies demonstrate that neuropilin supports this VEGF autocrine function in cells lacking KDR expression by stimulating the PI3-kinase pathway. These findings raise the exciting possibility that neuropilin functions either alone or in concert with other tyrosine kinase-linked receptors to transduce VEGF signaling in metastatic tumors. This involvement of neuropilin in breast carcinoma survival and the finding that neuropilin is expressed in metastatic but not in nonmetastatic tumor cells (19) suggest that neuropilin may be an important determinant of metastasis because it promotes tumor cell survival. The implications of this hypothesis with respect to both the mechanism of metastasis and therapeutic intervention are significant.

Acknowledgments

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Integrin Laminin Receptors and Breast Carcinoma Progression

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This review explores the mechanistic basis of breast carcinoma progression by focusing on the contribution of integrins. Integrins are essential for progression not only for their ability to mediate physical interactions with extracellular matrices but also for their ability to regulate signaling pathways that control actin dynamics and cell movement, as well as for growth and survival. Our comments center on the $\alpha6$ integrins ($\alpha6\beta1$ and $\alpha6\beta4$), which are receptors for the laminin family of basement membrane components. Numerous studies have implicated these integrins in breast cancer progression and have provided a rationale for studying the mechanistic basis of their contribution to aggressive disease. Recent work by our group and others on mechanisms of breast carcinoma invasion and survival that are influenced by the $\alpha6$ integrins are discussed.

KEY WORDS: Integrin; laminin; breast carcinoma; invasion.

INTRODUCTION

Cancer progression, defined as the sequence of events that enable tumor cells to become invasive and eventually metastatic, is among the most challenging and important problems in cancer biology and cancer medicine (1, 2). Although progression is a complex, multi-factorial process, the salient features are relatively simple. The metastatic odyssey of a malignant breast carcinoma cell, for example, from its genesis in the mammary gland to a distal organ requires that this cell acquire a motile phenotype to invade through tissue and gain access to the vasculature and lymphatics. In addition, this cell will apoptose outside of the nurturing environment of the mammary gland unless it adapts survival mechanisms. Thus, a simplified perspective on progression focuses on the ability of tumor cells to invade and survive. Indeed, much of contemporary cancer biology is linked to these two key parameters of progression.

The biology of breast carcinoma, as well as other carcinomas, is essentially an aberration of epithelial cell biology. The distinguishing features of epithelia are their polarized morphology, attachment to an underlying basement membrane, presence of specialized cell-cell contacts, and their capacity for rapid self-renewal, differentiation and death (3). These features are manifested in the epithelial and myoepithelial cells of the mammary gland. The basement membrane, in particular, plays a central role in the biology of epithelia and carcinomas. This thin sheet of connective tissue, which is comprised primarily of collagen type IV, laminins, entactin (nidogen) and proteoglycans, separates the epithelium from underlying stroma (4). Interestingly, much of our knowledge on the importance of the basement membrane in epithelial biology derives from studies on the mammary gland [reviewed in (5, 6)]. Such seminal studies established that the adhesive interactions of mammary epithelial cells with basement membrane laminins are

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essential for their differentiation and survival. The importance of these laminin-mediated interactions has stimulated interest in the receptors on epithelial cells that mediate laminin interactions and on the intracellular signaling pathways that these receptors influence.

Basement membrane interactions are also an important component of carcinoma progression. In fact, the breaching of the basement membrane by carcinoma cells is a defining event for malignant tumors (7). In addition, some carcinoma cells synthesize and deposit basement membrane components, especially laminins, and receptor-mediated interactions with these components generate signals that facilitate their migration and sustain their survival (8, 9).

LAMININS AND THEIR RECEPTORS IN MAMMARY EPITHELIA AND BREAST CARCINOMAS

The laminins, a large family of heterotrimeric glycoproteins, are major components of basement membranes. To date, more than twelve distinct laminins have been identified [for comprehensive review see (4)]. Although the importance of the laminins to epithelial and carcinoma biology is established, there is much to be learned about the expression and function of specific laminins. This situation is particularly evident for laminin function in mammary epithelia and breast carcinomas. Most of the pioneering functional studies on mammary epithelia used laminin purified from a murine sarcoma or a reconstituted basement membrane preparation from this tumor termed Matrigel, which is comprised primarily of laminin-1 and type IV collagen. Studies using laminin-1 and Matrigel have provided valuable information on the importance of laminin in mammary gland biology (5, 6). In addition, much has been learned about breast carcinoma migration and survival, as well as receptors that mediate these processes, using these reagents [e.g. (10, 11)]. Future studies, however, need to characterize the expression and function of specific laminins in the normal breast and in breast carcinoma in more detail. Recent studies on laminin-5, a laminin that appears to be particularly important for epithelial migration, exemplify the direction of future work in this area (12, 13).

Numerous surface proteins function as receptors for the laminins, including members of the integrin family, dystroglycan, a receptor tyrosine phosphatase, heparan sulfates and various other surface proteins (14, 4). Although integrins appear to be the 'preeminent' laminin receptors on most cells, including mammary epithelial and breast carcinoma cells, and they will be the focus in this review, the functional contribution of other laminin receptors to breast biology has not been studied extensively and warrants further investigation.

A recent review in this journal summarized integrin expression and function in mammary epithelia and breast carcinoma (15). As discussed in this review, the integrins expressed on the epithelial and myoepithelial cells of the breast include $\alpha2\beta1$, $\alpha3\beta1$, $\alpha5\beta1$, $\alpha6\beta1$, $\alpha6\beta4$, $\alpha v\beta3$, and $\alpha v\beta6$ (15). Several of these integrins function as laminin receptors ($\alpha2\beta1$, $\alpha3\beta1$, $\alpha6\beta1$, and $\alpha6\beta4$). Although most of these integrins probably contribute to breast cancer progression, it is the $\alpha6$ integrins ($\alpha6\beta1$ and $\alpha6\beta4$) that have captured the interest of many investigators because of their link to aggressive disease. This link was foreshadowed by the finding that high expression of the $\alpha6$ subunit in women with breast cancer correlated significantly with reduced survival times (16). In an analysis of 119 patients with invasive breast carcinoma, all of the patients with low or absent $\alpha6$ expression survived, while the mortality rate of the patients with a high level of $\alpha6$ expression was 19%. Of note, 30 out of 34 of the patients that presented with distant metastases were highly positive for $\alpha6$ expression. Although this study did not distinguish the relative contributions of the $\alpha6\beta1$ and $\alpha6\beta4$ integrins, subsequent work has implicated both of these integrins in breast carcinoma progression. For example, co-expression of $\alpha6\beta4$ and laminin in breast tumors has been correlated with poor prognosis (17). Also, expression of the $\alpha6\beta1$ integrin has been linked to the survival and metastatic potential of human breast carcinoma cells (18, 19). Given the potential importance of the $\alpha6$ integrins to breast cancer, the challenge ahead is to define the mechanisms by which these receptors promote progression.

BREAST CARCINOMA INVASION

Invasion, or the penetration of tumor cells into adjacent tissues, is one of the hallmarks of malignant tumors (7). In contrast to benign tumors that are encapsulated, invasive tumors have the potential to metastasize because of their access to lymphatics and the vasculature. Invasion can also result in patient morbidity and mortality in the absence of metastasis. For these reasons, understanding the process of

invasion is of obvious importance. The insightful observations of pathologists have taught us much about the nature of tumor invasion and have provided a foundation for more mechanistic studies (7). As mentioned above, breaching of the basement membrane by carcinoma cells is a defining event for malignant tumors because it facilitates their access to the vasculature and lymphatics. This observation spawned the 'three-step' model of invasion (20). This model implied that tumor cells attach themselves to the basement membrane and then secrete proteases that degrade localized regions of the basement membrane, enabling their migration into stroma. The most significant contribution of this model was that it identified critical components of the invasive process that could be studied in more detail, such as adhesion and proteolysis. As a result, we know much, for example, about receptors on tumor cells that mediate interactions with basement membrane components and proteases that degrade these components. We also know that the invasive process is more complex than envisioned originally. A significant advance in this regard was the realization that highly invasive carcinoma cells often lose contact with each other and exhibit a mesenchymal or motile phenotype that is distinct from normal epithelial structure (21–23). This realization led to the hypothesis that an epithelial to mesenchymal transition is a major component of the invasive process. Subsequent studies on the loss of cadherin-mediated cell-cell adhesion in invasive carcinomas established a mechanistic basis for this epithelial to mesenchymal transition (24–27). In addition, the advent of molecular cell biology has provided a new prospectus on signaling molecules and cytoskeletal dynamics that could regulate invasion. And, the realization that invading tumor cells must survive in foreign and often hostile environments has made the study of survival mechanisms an integral component of tumor progression. These advances are enabling us to approach the problem of invasion at a fundamental level.

The $\alpha6\beta4$ Integrin and Breast Carcinoma Invasion

In many epithelia, the $\alpha6\beta4$ integrin, which is a receptor for the laminins, mediates the formation of stable adhesive structures termed hemidesmosomes that link the intermediate filament cytoskeleton with specific laminins in the basement membrane (28). Although hemidesmosomes are most apparent in stratified epithelia, they also exist in breast epithelia but not in invasive breast carcinomas (29). Expression of the $\alpha6\beta4$ integrin, however, persists in many invasive cancers. In the breast, expression of $\alpha6\beta4$ is maintained in ductal carcinoma *in situ*, invasive carcinoma and in metastatic lesions as assessed by *in situ* hybridization (unpublished data). Moreover, as mentioned above, $\alpha6\beta4$ expression has been correlated with poor prognosis for breast cancer patients (17). Similar results have been reported for other solid tumors [reviewed in reference (30)]. Such studies have been substantiated by the findings that expression of $\alpha6\beta4$ in a $\alpha4$-deficient breast carcinoma cells dramatically increases the invasive potential of these cells (Fig. 1) (10, 31, 32). Other studies by our group provided evidence that the $\alpha6\beta4$ integrin promotes breast carcinoma invasion by stimulating lamellae formation and the chemotactic migration of breast carcinoma cells (Fig. 1) (33). Collectively, the available data support an important contribution of the $\alpha6\beta4$ integrin to carcinoma invasion (10, 31, 32, 34). More data are needed, however, to substantiate this finding. In particular, the involvement of $\alpha6\beta4$ in progression needs to be validated in transgenic models of breast cancer, and more studies are needed to validate the prognostic significance of $\alpha6\beta4$ expression in human breast carcinomas.

Cytoskeletal Interactions of the $\alpha6\beta4$ Integrin

The involvement of the $\alpha6\beta4$ integrin in invasion and migration conflicts with the established function for this integrin in mediating stable adhesive contacts in hemidesmosomes. In simple terms, migration is a dynamic process that requires the rapid formation and disassembly of adhesive contacts (35). The presence of $\alpha6\beta4$-containing hemidesomosomes would impede such dynamic events. A significant breakthrough, therefore, was our finding that the $\alpha6\beta4$ integrin can associate with F-actin and is localized at the leading edges of invasive carcinoma cells (36). Moreover, we demonstrated that $\alpha6\beta4$ actually mediates the migration of such cells through its ability to associate with the actin cytoskeleton and promote the formation and stabilization of filopodia and lamellae (36). This finding implied that the function and cytoskeletal association of $\alpha6\beta4$ in invasive carcinoma cells are distinct from its established role of anchoring epithelial cells to the basement membrane through its association with cytokeratins.

The observations on $\alpha6\beta4$ and migration raise two important issues: the mechanism by which hemidesmosomes are disrupted to enable $\alpha6\beta4$ to

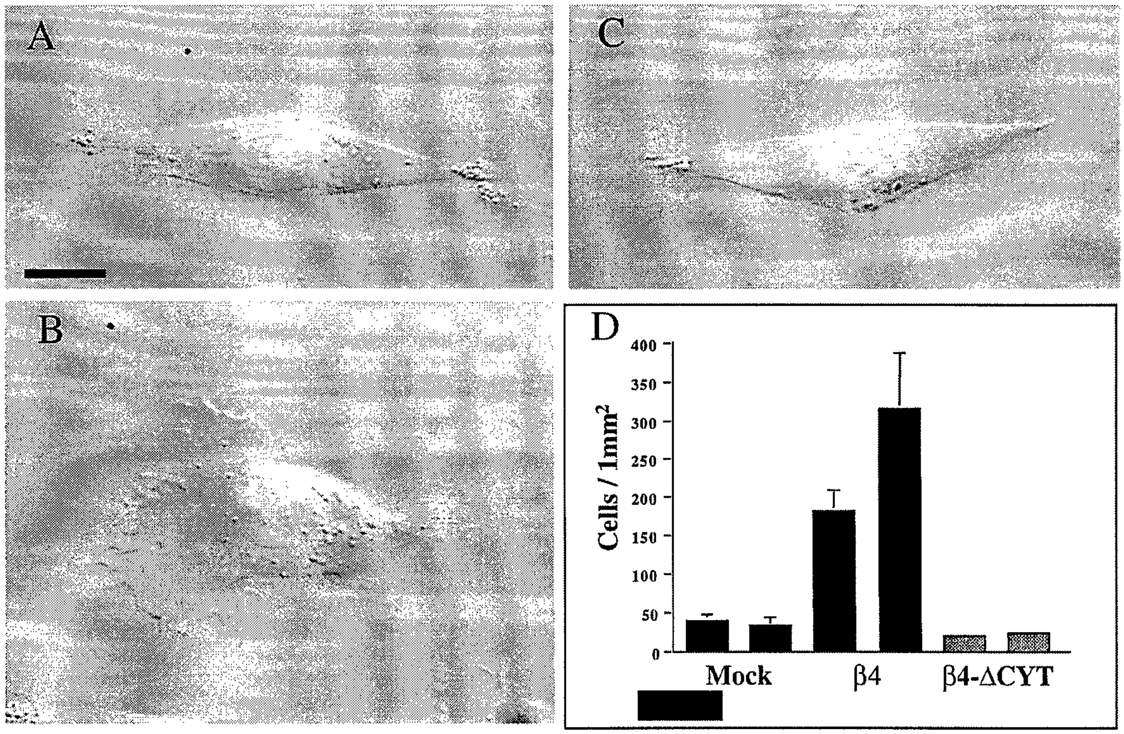

Fig. 1. Cooperation between $\alpha6\beta4$ integrin and growth factor signaling promotes lamellae formation and invasion (33). Human breast carcinoma cells (MDA-MB-435) that either lack expression of the $\alpha6\beta4$ integrin (C) or express this integrin (A, B) were plated onto coverslips that had been coated with 20 μg/ml collagen I. Cells were allowed to adhere for 2 hrs at 37°C and then treated with LPA for 5 min. (B, C) or left untreated (A). The cells were visualized using Nomarski DIC optics. Note the large lamella that is formed in response to LPA stimulation of cells that express $\alpha6\beta4$ (B) but not in cells that lack expression of this integrin (C). Also, note that $\alpha6\beta4$ expression does not result in lamellae formation in the absence of LPA stimulation (A). The influence of $\alpha6\beta4$ expression of the chemoinvasion of MDA-MB-435 cells using a standard Matrigel assay is shown in (D). Note expression of a cytoplasmic domain deletion of the $\beta4$ subunit ($\beta4$-ΔCYT) does not stimulate invasion.

interact with F-actin and the molecular characterization of the interaction between $\alpha6\beta4$ and F-actin. The latter issue has yet to be explored but recent studies have yielded insight into the mechanism of $\alpha6\beta4$ translocation to F-actin. Specifically, growth factors such as EGF can stimulate the chemotactic migration of carcinoma cells that form hemidesmosomes such as squamous-derived carcinoma cells (37, 38). The mechanism by which growth factors stimulate migration involves disruption of $\alpha6\beta4$-containing hemidesmosomes (37, 38), a process that liberates such cells from rigid anchorage to the matrix and facilitates their motility. Using EGF as prototypic growth factor, we demonstrated that growth factor stimulation of squamous carcinoma cells redistributes $\alpha6\beta4$ from hemidesmosomes to F-actin-rich lamellipodia and membrane ruffles, and that $\alpha6\beta4$ is required for chemotactic migration in response to EGF (Fig. 2) (38). An analysis of the signaling events involved in this re-distribution of $\alpha6\beta4$ from hemidesmosomes to F-actin revealed an essential role for protein kinase C-α. In addition, EGF stimulates phosphorylation of the $\beta4$ subunit coincident with $\alpha6\beta4$ redistribution (38). The causal role of $\beta4$ phosphorylation in disrupting hemidesmosomes and $\alpha6\beta4$ redistribution needs to be established. Nonetheless, these findings demonstrate that a chemotactic stimulus will not only disassemble hemidesmosomes but also promote the formation of $\alpha6\beta4$-containing lamellipodia and membrane ruffles, thus establishing a mechanism for the dichotomy of $\alpha6\beta4$ function in stably adherent and migrating epithelial-derived cells.

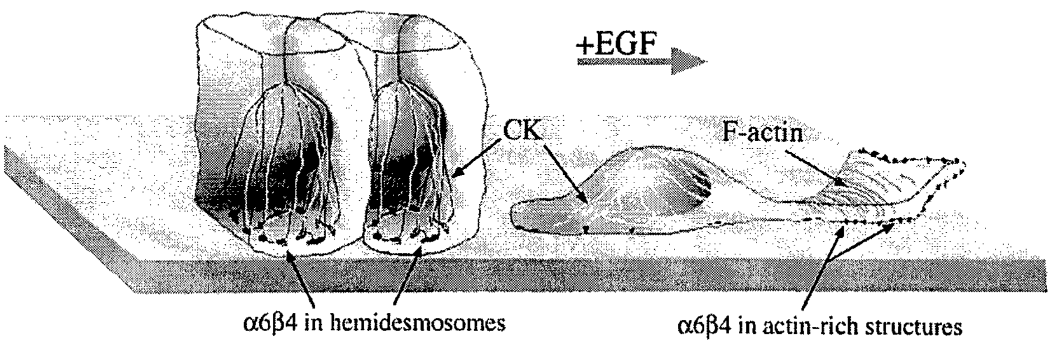

Fig. 2. Growth factor mobilization of the $\alpha6\beta4$ integrin from its association with cytokeratins (CK) in hemidesmosomes to F-actin in lamellae and lamellipodia. A chemotactic stimulus such as EGF can disassemble hemidesmosomes and promote the formation of $\alpha6\beta4$-containing lamellipodia and lamellae. These findings establish a mechanism for the dichotomy of $\alpha6\beta4$ function in stably adherent and migrating epithelial-derived cells. An important implication of this model is that chemotactic factors can drive the migration of invasive carcinoma cells by mobilizing $\alpha6\beta4$ and disassembling hemidesmosomes.

Signaling Properties of the $\alpha6\beta4$ Integrin that Promote Invasion

The ability of integrins to regulate intracellular signaling pathways has been established [reviewed in (39)]. The current literature abounds with studies on integrin-mediated regulation of signaling pathways that control cell growth, differentiation and survival, as well as cytoskeletal dynamics and cell migration. Moreover, it has become apparent that integrins often function in concert with specific growth factor receptors to execute these functions. A mechanistic understanding of invasion requires that integrins be identified that contribute to specific components of the invasive process and that the signaling pathways involved be elucidated. Such studies must incorporate the contribution of growth factor receptors, as well as those extracellular matrix components that interact with these integrins. Although a challenging task, considerable progress has been made in recent years, largely because of conceptual and technical advances in cell biology. Our comments here will focus on the contribution of $\alpha6\beta4$ integrin signaling to progression based on our interests and research. However, the reader should bear in mind that the signaling pathways that we highlight are regulated by other integrins as well and the contribution of these integrins to invasion needs to be considered.

A link between $\alpha6\beta4$-stimulation of breast carcinoma invasion and signal transduction was provided by our finding that this integrin activates phosphoinositide 3-OH kinase (PI3-K), a key signaling molecule, and that the activity of PI3-K is essential for invasion (10). Studies by Keely *et al.* (40) also established the importance of PI3-K in breast carcinoma invasion. PI3-K phosphorylates phosphatidylinositol (PtdIns) lipids on the 3′ position of the inositol ring, resulting in an accumulation of PtdIns(3)P1, PtdIns(3,4)P2 and PtdIns(3,4,5)P3 (41). Accumulation of D3 phosphoinositides at the plasma membrane recruits secondary signaling molecules or effectors that mediate the diverse functions of PI-3K (41). Given these considerations, it is important that the mechanism by which $\alpha6\beta4$ activates PI3-kinase and by which PI3-kinase promotes invasion be elucidated.

Interestingly, it appears that $\alpha6\beta4$ is able to stimulate PI3-K activity to a higher level than other integrins, at least in carcinoma cell lines (10). Although the mechanism by which $\alpha6\beta4$ or any integrin activates PI3-K has not been established, recent studies by Falcioni and colleagues have provided insight into this important problem. They reported that $\alpha6\beta4$ associates with erbB2, a receptor implicated in breast cancer progression (42), on the surface of breast carcinoma cell lines (43). Subsequently, this group made the important observation that both $\alpha6\beta4$ and erbB2 are required for the activation of PI3-K and the stimulation of invasion using a 3T3 cell model system (44). The implication of this finding is that the cooperation of $\alpha6\beta4$ integrin signaling with specific growth factor receptor signaling is required for PI3-K activation and consequent stimulation of invasion (Fig. 3). The task ahead is to define the mechanism of this cooperative signaling. One hypothesis is that $\alpha6\beta4$ potentiates growth factor receptor signaling by increasing the activation of a key signaling intermediate(s). Clearly,

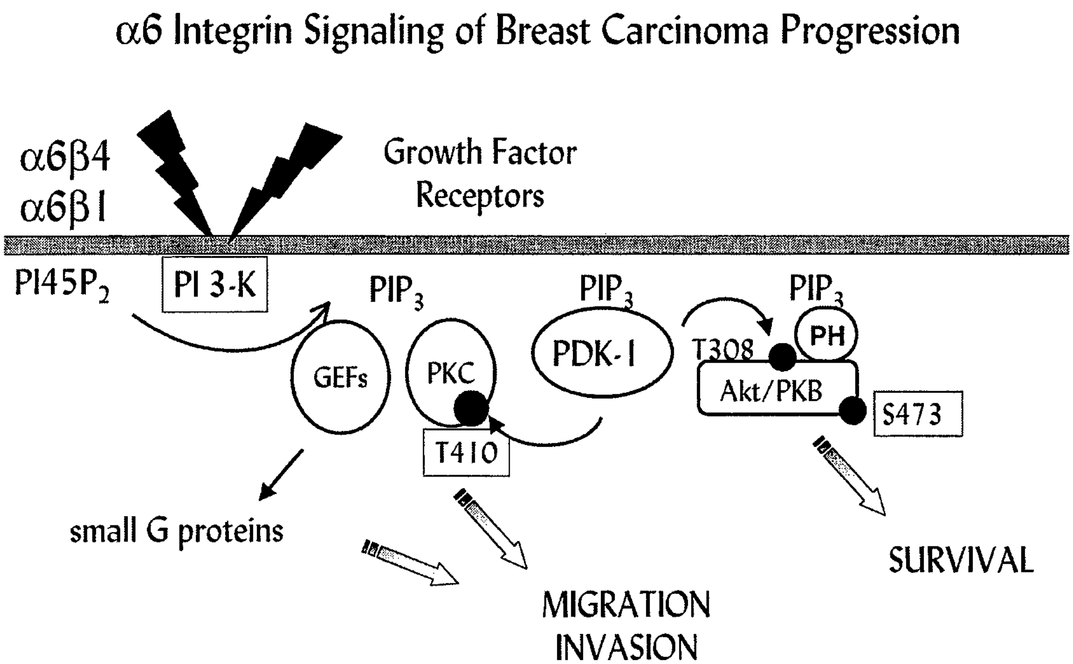

Fig. 3. Proposed model for how the $\alpha6$ integrins signal breast carcinoma progression. This model highlights the PI3-K signaling pathway (10). One important aspect of this model is that signaling from both the $\alpha6$ integrins and growth factor receptors is necessary for optimal activation of PI3-K as demonstrated in (44). However, the mechanism by which the $\alpha6$ integrins and growth factor receptors cooperate to activate PI3-K remains to be elucidated. Most likely, this synergy occurs at the level of signaling molecules that function downstream of these surface receptors and upstream of PI3-K. The ability of PI3-K signaling to promote invasion is hypothesized to result from the PI3K-dependent activation of small G proteins and specific PKC isoforms that regulate actin dynamics as discussed in (74). Also, the ability of the $\alpha6$ integrins to maintain the survival of carcinoma cells in adverse conditions (11, 18) results from the PI3-K dependent activation of the Akt/PKB kinase (65).

elucidation of this mechanism will be a significant advance not only because of the potential importance of $\alpha6\beta4$ in invasion but also because it will provide a paradigm for how integrin and growth factor receptors cooperate to drive the invasive process.

The mechanism by which activation of PI3-K promotes invasion is central to our understanding of carcinoma progression. The major function of this enzyme with respect to invasion appears to be its ability to regulate the cytoskeleton and cell migration (10, 40). Its role in survival will be discussed below. For this reason, the effectors of PI3-K that have the potential to regulate these functions are prime targets for study. In reality, however, little is known about the involvement of specific PI3-K effectors in invasion and how they contribute to the migration of these cells. The remarkable progress that has been made recently in understanding how signaling pathways, including the PI3-K pathway, regulate actin dynamics (45–47) should accelerate studies on carcinoma migration and invasion and perhaps reveal differences in signaling between normal epithelial and carcinoma cells. Interesting observations have already been made in this direction. For example, the small G protein Rac, a PI3-K effector, appears to be involved in the migration and invasion of breast carcinoma cell lines (10, 40). In contrast, Rac inhibits the migration of normal epithelial cells by promoting the formation of cell-cell adhesions (48). Another effector of PI3-K that is of paramount importance in both cell survival and cancer progression is the Akt/PKB kinase (see below). Although a role for this kinase in migration and invasion had been discounted (10, 49), recent work on Dictyostelium support a role for Akt/PKB in migration (50). It may be worthwhile, therefore, to re-evaluate the contribution of this kinase to carcinoma invasion.

The $\alpha6\beta4$ integrin also regulates another signaling pathway, apparently distinct from PI3-K, that is important for the migration and invasion of carcinoma cells. This pathway involves the metabolism of

cAMP. The $\alpha6\beta4$ integrin, in concert with growth factor receptors, stimulates the formation of lamellae and the consequent chemotactic migration of breast carcinoma cells, processes that are inhibited by intracellular cAMP (33). We observed that $\alpha6\beta4$ can reduce the intracellular cAMP concentration by activating a cAMP-specific phosphodiesterase, and that this activity is essential for the $\alpha6\beta4$-mediated enhancement of lamellae formation and chemotactic migration (33). Subsequent studies revealed that cAMP inhibits activation of the Rho GTPase in carcinoma cells (51), similar to the regulation of Rho in leukocytes (52). Moreover, these studies led to the important observation that $\alpha6\beta4$ stimulates Rho activity in carcinoma cells, and that Rho is essential for their migration and invasion. These results are of interest in light of other reports that both Rho and Rho kinase are important for tumor cell invasion (53, 54). Together, these findings suggest that $\alpha6\beta4$-mediated regulation of the Rho/Rho kinase pathway may be an important signaling component of carcinoma progression.

BREAST CARCINOMA SURVIVAL

Arguably, the ability of tumor cells to survive at sites different from their origin is the most important determinant of metastasis. If such cells can survive at distal sites, they have the opportunity to expand in number and form metastatic lesions. On the contrary, invasive and metastatic cells that are unable to survive present no threat to the patient. For this reason, an understanding of the mechanisms that enable the survival of metastatic cells is essential not only for an understanding of progression but also for the development of therapeutics.

Given the fact that carcinoma cells are essentially malignant epithelial cells, an understanding of survival mechanisms used by epithelial cells merits discussion. It has been known for some time that the attachment of primary epithelial cells to extracellular matrix (ECM) proteins is essential for their survival (55, 56). More specifically, integrin-mediated interactions with ECM proteins initiate signals that sustain survival (57). Survival is enhanced significantly by growth factor stimulation of attached cells, providing evidence that integrins and growth factor receptors cooperate to promote survival (57). The environment within the epithelium that supports survival, however, is progressively lost during malignant transformation, and especially as malignant cells become invasive and metastatic. Positional cues such as basement membrane anchoring and cell-cell adhesive contacts that provide survival signals in the normal epithelium are often absent in invasive and metastatic cancer (5, 58). Moreover, the environment encountered by invasive and metastatic cells is foreign and often pro-apoptotic. For example, one of the most formidable barriers to their survival is hypoxia (reviewed in (59)). The oxygen tension within many solid tumors is substantially less than that in adjacent normal tissue, presumably because of poor vascularization (59). The conclusion can be drawn from these considerations that invasive and metastatic cells must acquire mechanisms that maintain their survival outside the confines of the epithelium. Indeed, cancer progression can be considered an evolutionary process that selects for cells that exhibit the capacity for survival, among other properties (1, 2). One important implication of this hypothesis is that those cells that do survive will be the most aggressive because they have the capacity to survive in inappropriate locations and form metastatic lesions. In other terms, a strong selective pressure promotes the growth of metastatic cells that have evolved mechanisms for surviving in hostile environments.

The ability of tumor cells to recruit blood vessels, a process referred to as the 'angiogenic switch' is a major mechanism for sustaining the growth and survival of tumor cells (60). In fact, considerable evidence supports a direct link between angiogenesis and tumor progression (61). Another mechanism of survival that has not been investigated as intensively as the angiogenic switch is the ability of tumor cells themselves to turn on signaling pathways that promote their survival. The hypoxic regions of many solid tumors, for example, are poorly vascularized, yet some of the cells are able to survive and progress to metastatic disease (reviewed in (59) as the possible result, for example, of their secretion of growth factors that act in an autocrine manner to promote their survival.

The Central Role of PI3-K in Survival

Numerous studies have substantiated the importance of PI3-K and its effectors in promoting cell survival (41, 62). Moreover, it is worth noting that at least two of the genes implicated in cancer, Ras (63) and PTEN (64), impact the PI3-K signaling pathway. The key effector molecule in this pathway appears to be Akt/PKB (Fig. 3). PI3-K is essential for Akt/PKB activation because the D3 phosphoinositide products of PI3-K bind to its pleckstrin homology (PH) domain and recruit it to the cell membrane (41, 62).

These phosphoinositides also recruit another critical enzyme PI3-K-dependent kinase 1 (PDK1) to the membrane enabling it to phosphorylate Akt/PKB at threonine residue 308 (Fig. 3). This phosphorylation event stimulates Akt/PKB autophosphorylation at serine residue 473, which is the final step required for the stimulation of Akt/PKB kinase activity towards exogenous substrates (65).

The importance of Akt/PKB in cell survival has been associated with both its ability to inactivate key apoptotic molecules as well as its ability to stimulate anti-apoptotic signaling pathways [reviewed in (62)]. Specifically, Akt/PKB has been shown to phosphorylate and inhibit Bad, a Bcl-2 family member, and inhibit the activity of forkhead transcription factors, which play an essential role in Fas death receptor-induced apoptosis. Akt/PKB also phosphorylates and activates transcription factors implicated in diverse survival signaling pathways, including NF kappa B and CREB. The stimulation of Akt/PKB activity is associated with both growth factor receptor and integrin signaling, and optimal Akt/PKB activation is achieved as a result of the cooperative signaling that occurs between these two types of receptors (56).

Given the potential importance of Akt/PKB in the survival of both primary (66) and transformed (11) epithelial cells, the hypothesis can be formulated that cancer progression selects for cells that have the capacity to sustain Akt/PKB activation. This hypothesis is supported, in fact, by compelling genetic data. The PTEN tumor suppressor is a lipid phosphatase that dephosphorylates PtdIns(3,4,5)P3, a PI3-K product that is essential for Akt/PKB activation (64). Importantly, PTEN-deficient cells are resistant to numerous apoptotic stimuli and have constitutively elevated levels of Akt/PKB activity (67). Moreover, numerous cancers contain mutations or deletions in PTEN that could facilitate Akt/PKB activation [reviewed in reference (64)]. In addition to PTEN, the Ras GTPase is activated by mutation in a significant fraction of human cancers (63), and activated Ras can sustain activation of the PI3-K-Akt/PKB pathway (66, 68). The importance of this activation is suggested by finding that activated Ras can protect epithelial cells from the apoptosis that occurs when they are detached from matrix and deprived of integrin signals, a process referred to as anoikis (66). One implication of this finding is that activated Ras can promote cell survival in the absence of integrin signaling, a process, for example, that could enable tumor cells to survive in environments where integrin signaling is deficient because of the ECM composition.

Another mechanism that may contribute to the survival of carcinoma cells is Akt/PKB overexpression. Of the three Akt/PKB isoforms that have been described, Akt-2 has been shown to be upregulated in numerous cancers, including breast, as a result of gene duplication (69–71). Moreover, there is some evidence that Akt-3 activity may be elevated in aggressive breast carcinoma cancer cell lines (72). Although more data are needed to assess the impact of Akt/PKB overexpression on carcinoma survival, as well as the contribution of specific Akt/PKB isoforms, this clearly is an area of potential importance.

The probability that specific integrin and growth factor receptors contribute to carcinoma survival by stimulating Akt/PKB activity is high, based on the known ability of these receptors to activate Akt/PKB and their involvement in carcinoma progression. Moreover, this signaling mechanism could function together with genetic alterations in other signaling molecules, as well as Akt/PKB overexpression, to sustain elevated Akt/PKB activity in tumor cells existing in hostile environments as a means of maintaining their survival. The $\alpha6$ integrins are obvious candidates for contributing to the Akt/PKB-mediated survival of carcinoma cells based on their ability to activate this kinase, as well as their involvement in carcinoma progression.

The $\alpha6$ Integrins and Carcinoma Survival

Studies, particularly on breast carcinoma, indicate an important contribution of the $\alpha6$ integrins to carcinoma survival (16, 18, 19, 73). As discussed above, this function was foreshadowed by the finding that high expression of the $\alpha6$ subunit in women with breast cancer correlated significantly with reduced patient survival times (16).

To define the contribution of the $\alpha6\beta1$ receptor to breast cancer more rigorously, we developed a genetic strategy for eliminating expression of this integrin in metastatic breast carcinoma cells (18, 73). When these $\alpha6\beta1$-deficient cells were inoculated into the mammary fat pad of nude mice, primary tumor size was significantly diminished compared to the parental cells. Further analysis revealed that this reduction in tumor size resulted from the apoptosis of these $\alpha6\beta1$-deficient cells (18). More importantly, the $\alpha6\beta1$-deficient cells did not form metastases in the lung, as did the parental cells, because of their inability to survive in this organ (18). These data indicated that $\alpha6\beta1$ is essential for survival of breast carcinomas

in vivo. The mechanism by which the $\alpha6\beta1$ integrin promotes survival *in vivo* is not known but a reasonable hypothesis based on other studies is that this integrin is needed to cooperate with specific growth factor receptors for optimal activation of Akt/PKB.

Recent work by our group on $\alpha6\beta4$ integrin signaling has also highlighted the importance of the $\alpha6$ integrins in breast carcinoma survival and substantiated the involvement of Akt/PKB. This work derives from the finding, discussed above, that $\alpha6\beta4$ activates the PI3-K/Akt/PKB pathway in carcinoma cell lines (10). An implication of this finding is that $\alpha6\beta4$ should also promote the survival of these cells. In fact, we observed that expression of $\alpha6\beta4$ enhances the survival of breast carcinoma cells that have been deprived of both serum and matrix, conditions that normally promote detachment-induced apoptosis (11). Interestingly, however, this stimulation of AKT-mediated survival by $\alpha6\beta4$ is apparent only in carcinoma cells that contain inactivating p53 mutations. In fact, the deprivation of wild type p53-expressing cells of both matrix and serum actually triggers the caspase-3 mediated cleavage and inactivation of AKT and prevents $\alpha6\beta4$-mediated survival signaling (11). Collectively, our findings highlight the importance of $\alpha6\beta4$ in promoting the AKT-dependent survival of breast carcinoma cells. This survival function could be particularly important in late-stage carcinomas, which exhibit a high frequency of p53 mutations and which are more prone to survive in hypoxia (59).

The observation that the p53 tumor suppressor can inhibit Akt/PKB kinase activity is of interest in light of the finding mentioned above that the PTEN tumor suppressor can also inhibit cell growth by inhibiting Akt/PKB in a manner that is dependent on its lipid phosphatase activity (64). Together, our findings on p53 and the previously described activities of PTEN highlight the impact of tumor suppressors on integrin-mediated functions. Moreover, our demonstration that p53 inhibits $\alpha6\beta4$ survival signaling by promoting the caspase-dependent cleavage of Akt/PKB provides a mechanistic link between tumor suppressor function and the regulation of integrin signaling, similar to the phosphatase activities of PTEN.

SUMMARY OBSERVATIONS

This essay has affirmed the potential contribution of one class of surface receptors to the progression of breast carcinoma. Although more issues have been raised than have been resolved, a blueprint for future studies on the molecular cell biology of progression is emerging. First and foremost, progression appears to be a web of many strands. Surface receptors, the ECM, signaling molecules and the cytoskeleton are some of the major 'strands' that form the web of progression. Studies on one 'strand' inevitably lead to other strands as our work on the $\alpha6$ integrins has demonstrated. Another theme that is emerging is that the key functional components of progression such as invasion and survival may result from stimulation of a common signaling pathway. In this direction, the argument can be made that the PI3-K pathway is a major determinant of progression because the effectors of PI3-K signaling regulate both invasion and survival (Fig. 3).

One direction for future work is to define further the contribution of effector molecules to specific aspects of progression. There is much to be learned, for example, from studies on the small G proteins and specific PKC isoforms with respect to cytoskeletal dynamics, migration and invasion. Also, studies investigating how Akt/PKB isoforms are regulated, both positively by integrin and growth factor receptor signaling, as well as negatively by tumor suppressors, will clarify our understanding of the mechanisms by which breast tumor cells survive in hostile environments that normally promote apoptosis. From the clinical perspective, the mechanistic understanding of breast cancer progression that is emerging provides an array of targets for rational drug design.

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